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CANCER RESEARCH

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CANCER RESEARCH

VOLUME 12 JULY 1952 NUMBER 7

An Evaluation of the Cancer Problem*

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INTRODUCTION

The Association is now meeting in its forty-third annual conference for the purpose of reporting progress and exchanging ideas. It is not necessary for me to report that the Association is flourishing. The quantity and quality of the scientific papers, the growing membership, and the active committees all speak for a vigorous organization. Between annual meetings members and others are kept abreast of progress by the Association's journal, *Cancer Research*, which, with the continued interest and financial support of its sponsors and the guidance of an able editor, is also flourishing.

This association of research workers is different from most other scientific societies in that it is centered, not around a basic discipline, but around a disease process. Most members belong also to the special professional organization representing their primary field of training. In this Association we are united to conquer a disease. When this disease is brought completely under control, interest in the Association will rapidly wane. Its members will be happy to see the Association disbanded when it has fulfilled its mission, as did another national society recently when the disease with which it was concerned was conquered. The present flourishing condition of this Association is cause both of pride and of regret.

Cancer research is becoming progressively more highly specialized as the simpler problems are solved, as new technics are used, and as additional scientific disciplines enter the field. The problem of effectively communicating new ideas with one another is becoming a serious problem, which will grow worse. While this is deplored, it appears to be unavoidable, and it is an indication of activity if not progress. However, there is still one clear central point on which all minds in this Association meet: We are out to help control cancer, and as quickly as possible.

United as the members are for the conquest of a particular disease, and coming to the campaign from many disciplines, it is highly desirable that everyone clearly understands the problem. Effective attack depends on a clear understanding of the enemy. In a few minutes I should like to sketch broadly and, perhaps, too boldly an outline of the cancer problem as it appears to me—to leave the details with which our sessions are concerned for a few moments and look at the over-all picture. The analysis is my own; the president speaks only to you, not for you.

Specifically, I should like to restate the problem by dividing it into three parts: namely, an appraisal of its size and composition, a broad analysis of the amount and type of progress made to date, and comments on the significance of these observations for future work. The first of these will further be subdivided into three compartments each of estimated size, namely, proportion of cancer now controlled, the educational problem, and the part remaining for research.

SIZE OF THE CANCER PROBLEM

Although the distribution of cancer is worldwide, the present analysis of the size of the problem is, for convenience and greater accuracy, limited to the United States. We are told that present cancer mortality is over 200,000 per year and that it is increasing at the rate of approximately 3 per cent or about 5,000 per year due to a greater number of persons living into the older age groups. Specifically, 207,721 deaths from cancer and leukemia were reported in 1948 (1).

Both the mortality and the total number of

* Presidential address delivered before the 43d annual meeting of the American Association for Cancer Research, Inc., in New York, April 12, 1952.

cases of cancer are much larger than the 207,721 mortality reported on death certificates. In the vital statistics, cancer may be both over-reported and under-reported. The former is due to false diagnoses of cancer. It is known that this error is small—probably less than 5 per cent of reported cases. Under-reporting may be due (*a*) to deliberate omission on death certificates of cancers that had been correctly diagnosed; (*b*) to failure to report recognized cancer in persons where some other major disease such as pneumonia or heart disease is also present and is reported as the cause of death; (*c*) to failure to diagnose correctly some cancers which have produced symptoms; (*d*) to failure of diagnosis of clinically silent cancers; and (*e*) to failure to report cured cancers. Under-reporting in the vital statistics is believed to be

TABLE 1

TOTAL CANCERS IN THE UNITED STATES IN 1948 (ESTIMATED)

Reported cancer deaths	207,721
Estimated unreported cancer deaths	41,500
Estimated cures	23,000
Estimated subclinical cancers*	14,500
Total	286,721

* Exclusive of occult prostatic carcinomas.

much greater than over-reporting, especially in internal types of tumor which are difficult to diagnose. While the situation is not so bad as it was some years ago, it is far from perfect, as anyone can verify by performing necropsy examinations in cases in which the death certificate had already been completed. Under-reporting due to causes (*a*), (*b*), and (*c*) combined is believed to be about 20 per cent of all tumors, to cause (*d*) about 1 per cent of all deaths, and to cause (*e*) it varies greatly with the primary site of the tumor.

In any population the total number of cancers occurring within a given period of time must equal (*a*) the deaths with overt cancer whether diagnosed and reported or not, plus (*b*) the number of complete cures, plus (*c*) the subclinical or occult cancers present in those persons who died of other causes during that time.[1] The national reported figure of 207,721 cancer mortality fails to include many cases in group (*a*) (because of failure in recognition and reporting), all of group (*b*), and nearly all in group (*c*). That figure is, therefore, a great understatement of the national cancer mortality and morbidity.

[1] The number of cases first diagnosed in an earlier period of time but dying in the period under study is balanced by those first diagnosed in the period under study but dying later, since the improvement in cure rate over short periods of time is small in proportion to all cases.

An attempt has, therefore, been made to estimate the total number of cancers occurring in the United States in 1 year (Table 1). On the basis of personal necropsy experience and from reports in the literature, it is estimated that the failures in diagnosis and correct reporting together equal 20 per cent of the total reported, or a figure of about 41,500 cases for 1948. According to this method of calculation the total mortality was nearly 250,000 in 1948. To this figure must be added the total cases cured during the year. This number is estimated at 23,000, a figure which is obtained by multiplying the number of reported fatal cancers of each type in Table 2 by the estimated percentage

TABLE 2

REPORTED CANCER DEATHS IN THE UNITED STATES (1948)*

Rank	Site	No. reported	Per cent of all cancers
1	Large intestine	33,694	16.2
2	Stomach	26,215	12.6
3	Mammary gland	19,162	9.2
4	Uterus	17,120	8.2
5	Lung	16,331	7.9
6	Prostate	11,758	5.7
7	Liver and biliary	10,598	5.1
8	Lymphatic†	9,129	4.4
9	Pancreas	8,234	4.0
10	Urinary bladder	6,278	3.0
11	Intracranial	5,440	2.6
12	Ovary	5,287	2.5
13	Esophagus	3,681	1.8
14	Skin	3,568	1.7
15	Kidney	3,342	1.6
16	Mouth	3,150	1.5
17	Bone	2,366	1.1
18	Pharynx	1,994	1.0
19	Larynx	1,868	0.9
20	Mesentery, etc.	1,409	0.7
	Subtotal	190,624	91.7
	All other types	17,097	8.3
	Total	207,721	100.0

* Modified from reference (1).
† Exclusive of lymphosarcoma.

of cure for each of these types, given in Table 3. Finally, to these two figures must be added the silent or subclinical cancers present in all persons who died during 1948. On the basis of necropsy experience this figure is believed to be about 1 per cent, if the disputed occult prostatic cancers are excluded. Since 1,444,337 deaths from all causes were recorded during 1948, it is calculated that about 14,500 subclinical cancers were present but not reported on death certificates. The sum of the four figures, in Table 1, is 286,721 cancers for the year 1948. This total is more probably an underthan an overestimation, but it has value nevertheless in characterizing the total size of the cancer problem.

The figure for cancer prevalence, i.e., the total number of cases present at any time, is much greater than the annual total, because average survival is more than 1 year.

Composition of the Cancer Problem by Types

While all cancers may have in common one fundamental pathological cellular process, that of neoplasia, from other points of view they are different diseases. On the basis of frequency, causation, life history, degree of inherent malignancy, symptomatology, problems in diagnosis, method of treatment, and prognosis they suggest different diseases. A quantitative evaluation of the cancer problem should contain an analysis according to localization. This is here done in two different materials.

First, an analysis of the 207,721 cancer deaths reported on death certificates in the United States in 1948 shows that 91.7 per cent of the total were accounted for by twenty kinds of cancer given in Table 2. The other dozens of varieties of cancer together comprised only 8.3 per cent of the total. There are, therefore, comparatively few important types of cancer from the point of view of numbers. The numbers and relative proportions of each type are found in the table.

In the recording of cancer in the vital statistics, the classification used is far from perfect. For example, soft tissue sarcomas are not listed separately but are apparently included with the carcinomas of each organ site. Also, cancers of the liver and biliary system are combined, despite their natural occurrence under different conditions, elevating the combination to an unnaturally high position. Furthermore, tumors of the mesentery and peritoneum combined are in twentieth rank; since it is known that primary malignant neoplasms of both are unusual, this category must contain many metastatic tumors which have erroniously been reported as primary. In a tabulation of this type, skin cancers occupy a depressed position because of a high clinical cure rate. Internal tumors of types difficult to diagnose during life such as pancreatic, intracranial, renal, and hepatic tumors stand abnormally low. Under-diagnosis of tumors of some types results in others occupying an abnormally high relative rank. In this category are found some of the external and accessible tumors which are easy to diagnose. Such neoplasms occupy a relative rank in the vital statistics higher than they ever do in necropsy data where the internal tumors are more accurately represented. It is believed, for these reasons and others, that the data based on necropsies and given in Table 3 more accurately represent the true site situation than do those in Table 2.

Post mortem examination of populations is still the best method of determining cancer mortality, and even morbidity, if two provisions are met: that good clinical records be available to provide the data on the cancers previously cured in those

TABLE 3

COMPARISON OF PRESENT CONTROL WITH THE EDUCATIONAL AND RESEARCH PROBLEMS IN CANCER

1	2	3	4	5	6	7
Rank	Size	Per cent of all fatal cancers	Present cures (per cent)	Maximum cures with available knowledge (per cent)	Size of the educational problem (per cent)	Size of the research problem (per cent)
1	Stomach	12.2	5	35	30	65
2	Large intestine	10.5	15	65	50	35
3	Lymphatic	8.5	< 1	1	0	99
4	Lung	8.4	5	50	45	50
5	Uterus	7.8	20	75	55	25
6	Intracranial	5.8	10	20	10	80
7	Prostate	5.3	7	25	18	75
8	Mammary gland	5.2	20	75	55	25
9	Pancreas	4.8	< 1	5	5	94
10	Urinary bladder	3.5	15	30	15	70
11	Gall bladder	2.4	< 1	5	5	94
12	Ovary	2.2	15	30	15	70
13	Skin	2.2	85	98	12	2
14	Soft tissue sarcomas	2.1	15	25	10	75
15	Oral cavity	2.0	15	40	25	60
16	Esophagus	2.0	3	20	17	80
17	Kidney	1.9	10	20	10	80
18	Larynx	1.6	20	75	55	25
19	Bone	1.5	5	15	10	85
20	Liver	1.4	< 1	2	2	97
	All other types	8.7	10	25	15	75

persons and that the necropsied population be comprehensive and relatively unselected as to disease. These conditions sometimes obtain in large charity hospitals in regions where there is no competition by other institutions and specialists for selected types of cancers whose loss would unbalance the material; where all persons in that economic group die either in that one hospital or at home; and where the necropsy permit rate is high and unselective. Here the data pertain to the indigent of the area, and the principal factor which prevents the conclusions from applying to the entire population is the relatively small difference in incidence of some types of cancer associated with economic and social factors. Under the best necropsy conditions, data can be obtained on practically all cancers in the three categories, namely, fatal, cured, and subclinical, to provide a reasonably accurate picture of the cancer problem. Such data are unexcelled for determination of relative organ frequency, and, if the other necessary demographic data for the population are available, they may also be used to calculate incidence.

It has been my good fortune to have access to the results of two large series of necropsies which appear to be excellent for studying the site composition of the cancer problem. One is a series of over 8,000 examinations in the Department of Pathology of the University of Chicago. These data are unbalanced by several known selective factors (chiefly high specialization of some of the clinical staff), but they serve as an excellent general source of information. The other series of 35,293 necropsies is from the Los Angeles County Hospital where the material meets most of the criteria mentioned above.[2] The demographic data in this population will be given in a subsequent publication dealing with the racial aspects of this material. The following analysis on cancer localization is based on these necropsies.

The twenty most common types of cancer found in this group of 35,293 necropsies are given in Table 3, together with the relative proportions of each (in per cent). Together these twenty varieties of cancer comprised 91.3 per cent of the 6,072 malignant neoplasms found in the series. Thus, they represent the great bulk of the cancer problem from the numerical standpoint. The dozens of lesser varieties of cancer together constituted only 8.7 per cent, a situation comparable to that in the vital statistics. From a similar analysis of the tumors in 8,000 necropsies in our records at the University of Chicago, and from a study of reports in the literature, it appears that these data represent fairly closely the relative organ site distribution. In different series of published necropsy data there may be shifts in the relative position of some of the tumors, depending on methods of classification and tabulation, and on rates of cure; but these shifts are unimportant for present purposes, and they do not appreciably affect the deductions that follow.

In the two sets of data presented in the tables, namely, necropsy data and the U.S. vital statistics, differences are seen in the proportions and relative positions of some of the tumors. The internal tumors which are difficult to diagnose clinically are relatively less numerous in the vital statistics than in the necropsies. Obviously they are under-diagnosed in the former. For example, intracranial and pancreatic tumors are far below their expected frequency. These two types of tumor are among the most difficult to accurately diagnose clinically, and they rank high among neoplasms which the pathologist discloses at post mortem examination.

From the listings given in Tables 2 and 3, research workers can tell at a glance the relative numerical importance of the tumor which they are studying, and grantors of research aid can tell whether their support programs are well balanced. Of course, in most instances these points are unimportant, as the investigators are studying the nature of the neoplastic process itself, a phenomenon possibly common to all neoplasms.

Amount of Cancer Control Now Prevalent

Having estimated total cancer mortality as well as morbidity in this country and its distribution by organ and tissue sites, let us next consider what proportion of this problem has been solved in comparison with what remains to be done.

In the fourth column of Table 3 is given an estimation of the present cures for each of the twenty major types of cancer. By cure is meant permanent eradication of the disease and not merely 5-year survival. These figures were arrived at from an integration of my own observations, reports in the literature, and from consultations with many colleagues, both specialists in medical centers and general practitioners. These figures are not the results of medical centers or for selected cases. They are believed to represent, within reasonable limits of error, the national situation—the treated and untreated, the early and the late, the highly malignant and those of lower inherent danger, and the occult, the unrecognized, and the diagnosed cases of cancer. These figures may be challenged by

[2] The author is deeply grateful to the Research Committee of the Los Angeles County Hospital Staff for permission to study this material and for help of the Pathology staff, especially Dr. E. M. Butt.

some critics as too optimistic and by others as unduly pessimistic, but in either event they could still serve the purpose of helping to characterize the cancer problem in terms of present cures and the educational and research problems that remain.

It is seen that the estimated percentages of cure range from a high of 85 for skin cancer (including basal-cell carcinoma but excluding malignant melanoma) down through 20 per cent for cancers of the larynx, uterus, and mammary gland, to a low of less than 1 per cent for cancers of the pancreas, gall bladder, and liver and in the malignant lymphatic diseases. If claims of occasional cures of localized lymphosarcoma are accepted as valid, there is today no longer any category of tumor with a mortality of 100 per cent. This is an improvement over the situation of one decade ago, but it is far from satisfastory. If the figure of 286,721 total cancers for 1948 is used and the cures as calculated numbered 23,000, the cures were only 8.0 per cent of all types combined.

It should again be emphasized that the results of treatment of some tumors in selected series of cases and in some medical centers are far better than those estimated as national averages. Also, the factor of palliation has not here been considered as it will be later. Furthermore, the situation is a little better now than it was in 1948, and any of these figures could become obsolete overnight in the event of improvement.

In summary, the figures in column 4 of Table 3 give an estimation of the proportion of the problem already solved, for each major type of cancer, if cure alone is used as the criterion.

Size of the Educational Problem

It is well known that present actual rates of cure in many cancers lag behind the theoretical, i.e., the levels that would prevail if all available knowledge was used. The difference between the two levels, actual and theoretical, represents a complex problem composed of educational (both lay and professional), psychological, economic, and other factors. It is believed that the largest of these is the educational factor, so that the group as a whole is, for convenience, designated by this term. How big is this educational problem? What is the difference in size between actual and present theoretical cure rates?

In the fifth column of Table 3 are given estimations, arrived at by methods similar to those used for the fourth column, on what the mortality would be in each of the twenty major types of cancer if all reasonably practical knowledge now available on detection, diagnosis, and treatment could be fully applied to the entire population. It presupposes that all feasible knowledge on cancer case detection could be put to use; that all persons would comply with recommendations on frequent screening regardless of convenience and other considerations; that all individuals with first symptoms would promptly go to a doctor; that this physician or a consultant would quickly and competently carry out the best known diagnostic and therapeutic measures; and that the economic where-with-all for these steps would be available to every person.

Actually, some of the cure frequencies might be higher than is indicated by the figures given in the table, provided some detection, diagnostic, and preventive measures now known but appearing to be impractical from the economic and psychological points of view would be used. Where, for example, would the line be drawn on the financial support of cancer detection? Would it be at ten, one hundred, one thousand, ten thousand, or one hundred thousand dollars per case? How long would the housewife, business executive, farmer, or factory worker continue to go for screening examinations in the face of negative reports? Would he stop at age 60 years after having had 40 negative reports since age 40 at the rate of two per year, or would he continue to comply with recommendations? This example is exaggerated to show some of the psychological and economic factors involved. Grave medical factors also are involved. Should, for example, all gall stones be removed even if otherwise harmless in order to prevent possible subsequent cancer in this organ, and, if so, should this procedure also be applied to the uterus after completion of child bearing, and in other sites?

It is seen, by comparing the figures in columns 4 and 5, that a considerable improvement in the percentage of cures of most cancers is theoretically possible. On the average, cures could, if the figures are correct, be more than doubled. The figures given are estimates. Some critics would raise some figures and lower others, but in either event they would still serve to stress the gains that could be made.

The differences between the present theoretical and the actual rates given in column 6 (column 5 minus column 4), represent the size of the educational problem for each class of cancers. In some tumors it is large; in others, small. It is probable that the efforts here collectively called educational would yield the highest rewards in pulmonic, large intestinal, uterine, mammary, and laryngeal cancers and least in lymphatic, pancreatic, biliary tract, and hepatic tumors.

The figures show that the educational problem is nearly twice as large, in terms of human lives, as are the curative achievements at this time.

Before leaving this so-called educational problem, it should be indicated that research enters into this area also. It is not purely educational. For example, some of the psychological, economic, and professional inertias to co-operation in case detection could be overcome if simpler methods than those now available could be developed. At the present time different screening tests must be carried out for each of the twenty major types of cancer and for others. For some no screening methods are available. If some simple and accurate chemical, serological, immunological, or other type of test could be found which would screen for all types, the problems would be greatly simplified. And if some test could be found for the pre-cancerous state, Utopia, if such can be applied to cancer, would be here. It is not unreasonable to believe that such a test will some day be found. It should be a major objective.

The improved control of cancer by earlier diagnosis and treatment using presently available methods is extremely difficult from many points of view, including those of patient, the natural course of the disease, and the physician. It requires, for example, almost superhuman ability on the part of the physician. Using the figure of nearly 300,000 new cases per year, it is easy to calculate that each practicing physician sees on an *average* only two new cancers per year. Each of the major varieties may present a number of different clinical pictures, and there are dozens of less common kinds of cancer. Therefore, the physician must learn, remember, and be able to recognize at need literally scores of diverse clinical syndromes. Most of these he will either never have need for or use only a few times in the course of a lifetime. But he is expected to have them all on tap in his mind. Compared with the infectious diseases, for example, there are many more clinical types of cancer, and the penalty for delay in prompt diagnosis and first attack treatment is usually much greater. It is obvious that simplified tests for screening and diagnosis are urgently needed; that, failing these, therapy for the disseminated stages of the disease is imperative; and that otherwise the most promising possibility of control would seem to be by prevention. It is probable that the challenge to our best efforts will continue for some time.

It should be noted that the educational problem is not a simple unit but one with many facets. It has a large component in lay education. At the graduate medical level it is closely related to the problem of giving the best service to the population. At the undergraduate level it is related to the difficult financial problems confronting medical schools. At the scientific level it is related to many research problems including those of personnel and materiel.

In any event, the educational problem is large, and it is important. Efforts in this area should be highly rewarding in terms of lives saved. Our best wishes and our help go out to that great body of men and women who are engaged in this campaign.

Size of the Research Problem

If all available knowledge on the eradication of neoplasms could be applied, a large mortality would still remain. This represents the research problem confronting both clinicians and laboratory workers. Its size is exemplified by the difference between complete control, or 100 per cent on one hand, and the sum of the present cures and the educational problem (i.e., columns 4 plus 6 of Table 3) on the other. The estimated size of the research problem so calculated for each cancer is given in column 7. It varies from only a few per cent in the case of skin carcinomas to nearly 100 per cent in the malignant lymphatic diseases. An enormous amount of investigation has already been done on some of these tumors, as, for example, the malignant lymphomas, so that, in terms of the over-all picture, much less than 100 per cent of the problem remains; but, in terms of human mortality, that is the figure remaining to be eliminated.

This is the area with which this Association is primarily concerned. Here is the size of our enemy. Anyone who chooses may calculate the approximate size of the research problem of the particular tumor in which he is interested in actual mortality from column 7 of Table 3 and the appropriate figure in Table 2.

It is clear that the ultimate conquest of cancer is not only a big therapeutic and educational problem, but a large investigative problem as well. The problem remaining for research is more than 6 times the cure rate now prevailing, and more than 3 times that now theoretically possible under optimum conditions of education and co-operation. By the method of calculation here used, approximately two-thirds of the entire cancer problem still remains for research.

It is obvious that, as progress is made in research in therapy, the educational problem is automatically increased by a corresponding amount. In Table 3 migration is from right to left. In other words, as the size of column 7 is reduced, column 6 is proportionately increased until such a time as the improvement shows up in column 4.

COMMENTS ON CONTROL, EDUCATION, AND RESEARCH

The cancer problem in terms of morbidity is seen to be large, reaching a figure of nearly 300,000 cases per year. While control of some types is well along, in others it has hardly begun. A large educational effort, both lay and professional, by utilizing all presently available knowledge could result in a considerable improvement in control of some types of cancer but not of others. The residual mortality, after full application of all control knowledge now available, represents the magnitude of the research problem. This problem is small in the case of some types of cancer and large, approximating 100 per cent, in others. An expression of the size of the problem in terms of mortality is not entirely fair, because it fails to take into consideration the enormous amount of information not yet translated into application. However, by using this crude method, the present state of the problem can be expressed about as follows: one-tenth controlled by cure, two-tenths remain as an educational problem, and seven-tenths remain for research.

Up to this point, the cancer problem has been over-simplified in an attempt at precise expression. It has been presented only in quantitative terms by figures, whereas it is that but much more also. Qualitative differences also exist between what has been done and what remains. In terms of both numbers and complexity the easiest percentages have been conquered. The cures have been largely in the localized, external, and accessible cancers; the disseminated and less accessible remain. With research, however, problems have a way of changing, and what is difficult today becomes easier tomorrow. That is the purpose of research and is its goal. New information in contiguous fields may soften or make gaps in what were sheer blank walls barring advance. New technics and knowledge, like great leaders, have a way of appearing in crises, provided effort is being exerted. It is probable that what has happened so often in the past will continue in the future. We must be discouraged neither by the qualitative nor the quantitative aspects of the problem. Both can be overcome.

Progress in the past has been slow in human cancer, as in tuberculosis and some other diseases, but it has been steady. Decade by decade things get better. Progress has consisted of eroding away a few per cent from the mortality of one kind of tumor this year and a few per cent from another type at a later time. The sum of these small victories adds up to the impressive figures totaled for some cancers. Progress of this type can be expected to continue. It is slower than we would like, but it is acceptable, since it could eventually infiltrate and eliminate the mortality problem. This solid progress in no way precludes the possibility of sudden, dramatic advances.

In this connection arises one of our most important and difficult problems in public relations. It is that of explaining progress of this creeping substitution type to the public. To the initiate, progress in terms of a few per cent is apparent; to the others it is not. We are fighting for small percentages in each type of cancer and are grateful for the sum of numerous small conquests. Decade by decade they add up to perceptible, if not entirely satisfactory, sums. To lay persons such progress is not apparent, or, at least not so evident as was the sharp reduction of deaths from pneumonia with the introduction of new chemotherapeutic and antibiotic agents a few years ago. In cancer they see mortality continue among their friends and relatives apparently unabated. Even many of the victories are unknown to them, because the correct diagnosis, even in cured cases, may be given to only a few close relatives. Continual education based on accurate data is needed in this area if the confidence and support of the public are to be maintained.

ANALYSIS OF PAST PROGRESS

Having measured past progress, one wonders how it was achieved and what it teaches for the future. The estimations of present percentage of cures as given in column 4 of Table 3 for each of the twenty major kinds of cancer are a measure of creative thinking and skillful effort already translated into practice. Who is responsible for those victories? The answer is that many disciplines have contributed. For example, those who have aided in education, research, prevention, diagnosis, classification, understanding of the vagaries of the life history and behavior of cancers, and many others have made the results possible.

The direct credit for cures must be given almost entirely to two groups, namely, surgeons and radiologists. Ranking with them are the physician who suspects and establishes the diagnosis, the diagnostic roentgenologist, and the pathologist. Also in direct line of the attack are the various endoscopists, the gynecologist, dermatologist, urologist, neurologist, hematologist, otolaryngologist, and others. There is evidence that some cancers are being diagnosed earlier, and here public health workers, educators, and publicists are given credit.

Surgeons and radiologists are the first to admit that they accomplished those cures with the direct and indirect efforts of others. Surgeons give credit to many factors for their continuing improve-

ments in cancer therapy. Among these, better methods of diagnosis, pre- and post-operative care, anesthesia, and operative technics rank high. Control of infection by antibiotics and chemotherapy and of shock by transfusions is given much credit. Radiologists cite improvements in equipment and radiation technics, in classification and study of radiosensitivity, in understanding of the natural disease, and in supportive therapy by antibiotics and transfusions as important factors in their successes.

Behind these therapeutic victories, therefore, are a long list of basic scientific researches. High among these more remote but essential discoveries stand those of Landsteiner on blood groups, Fleming on antibiotics, Domagk in chemotherapy, Roentgen on x-rays, and the Curies on radium, to name only a few.

Up to this point progress has been measured chiefly by cure. This criterion is too narrow to leave unqualified. It fails, for example, to give credit to palliation. Enormous and gratifying progress has been made in prolonging life in usefulness and comfort by chemotherapeutic, endocrinological, surgical, and radiological palliative measures. Prolongation of active life from age 60–70 years in good health in cases of prostatic cancer by anti-androgen therapy is a heartening achievement, even though it is not reflected in mortality tables. In fact, under such conditions the difference between cure and palliation loses its importance. Furthermore, research that results in better palliation is significant because of the possibility that by becoming very slightly more effective it will be curative. Many disciplines have contributed to palliation.

In addition to cure and palliation, prevention should be mentioned. As an ultimate objective, it is unthinkable that anyone would settle for less than complete control of cancer in the population. Complete control of any disease can, theoretically, be achieved by prevention, by cure, or by both. The great victories in modern medicine, particularly in the communicable diseases, have come by prevention. Some cancers are already being prevented, and research has disclosed a large amount of information on carcinogens and carcinogenesis which may eventually be used in further prevention. Evidence for the prevention of some tumors is most clear in certain occupational groups where hazard has existed which has now been eliminated. Cancer is also being prevented in other areas where success is harder to measure. Who can say how many cancers have been prevented by gynecologists, dentists, dermatologists, surgeons, and others? It is known, for example, that some neoplasms arise in scars of old wounds in which there had been delay in healing but not in those that healed by primary intention. Good surgery, aided by control of infection and skin grafting, is probably preventing a number of cancers. The same may be said for other fields.

A cancer that has been prevented is not spectacular. It does not appear on operating room schedules, on morgue tables, or in saddened hearts. Its nonoccurrence can only be detected by special statistical methods. Knowing this, who would dare say that statistical tables in cancer are dry reading? Yes, the statistician also has a place in cancer. From analyses of the conditions of natural occurrence of the disease he can reveal clues to etiology, which, after having been definitely established, can lead to further prevention.

Even the combined standards of cure, palliation, and prevention do not adequately measure progress. They fail to include a tremendous fund of knowledge known about the disease. Here most of the biological and physical sciences have contributed. Among these, biochemistry, nutrition, genetics, virology, endocrinology, histology, physiology, pharmacology, and others should be mentioned. Some of this information is proximate to cancer problems and some appears remote, but it is all part of the foundation on which future developments can take place. Much of it requires only slight additional study or modification to be applied to the human problem. The mammary milk agent, and some of the contributions of genetics and chemical carcinogens, among many others, fall into this category.

Finally, a tremendous reservoir of knowledge on life and disease processes in the life sciences and elsewhere is available to cancer. We are not in this thing alone, although the direct responsibility is ours.

Implications for Future Research

What do the lessons of the past indicate as the most promising method of attack and the most probable source of the next major contributions? The answers to these questions indicate where research support should be given.

The past teaches that the achievements in column 4 came in two stages. First, some basic fact was discovered, usually in a laboratory, and then this information was applied to human problems by clinicians. In this application cancer often did not profit more than other diseases. Cancer surgery advanced with surgery in general but not disproportionately so. Cancer surgery did not surpass vascular, neurological, traumatic, or other forms of surgery. Cancer radiation therapy did develop

faster than radiation therapy in other diseases, but it did so because of the special susceptibility of the disease; but radiation therapy did not develop faster than did diagnostic roentgenology.

Lest it be thought that clinical research has had its big day and has little more to offer, consider some achievements of the past decade in the management of disseminated forms of cancer. Some cancers of the prostate, thyroid, and mammary gland respond to measures that were unknown 10 years ago (anti-androgen, radioactive iodine, and hormone therapy, respectively). Two deductions of wider application can be made from these examples: The disseminated forms of cancer are susceptible to attack, as were the localized forms; the control of disseminated cancer need not be an all-or-none phenomenon, but progress can be made piecemeal and by different modalities.

No one can predict from whence the next basic discoveries applicable to cancer will come. There was no way of forecasting that the fundamental discoveries already mentioned, namely, those of Landsteiner, Fleming, Domagk, Roentgen, and the Curies and others would prove so important to cancer. They were not made in cancer research laboratories, although they might have been, had more cancer laboratories existed in those days. The important point is that they were good, original, basic research.

The question is raised as to what is basic research. As here used this term refers to investigation so fundamental and substantial as to serve as the basis or foundation for future work and construction. It has to do with idea and attitude and not technic or materials. Research may be basic whether done on mice or men, on cancer or noncancer, on ordinary carbon as well as on radio-carbon. The "so what" type of research done on Drosophila or an enzyme system is no more basic than a poor investigation on human cancer. It is the idea that is fundamental and not the technic or material.

The main lesson of the past seems to be that good basic research should be supported, regardless of whether or not it appears at the moment to apply to cancer. However, cancer funds are not large enough to support all, or even very much, of all existing or desirable basic research. A line must be drawn, and the question is, where. Time and space do not permit more than a superficial examination of this important problem. It appears that if basic research can be done directly on cancer and of as good scientific quality as with other materials and problems, then first priority should be given to such cancer problems. It is my opinion that basic research of the best quality can today be done directly on cancer problems. Therefore, if a choice must be made between proposals in basic research, one closely related and the other distant to tumors, the former should have priority, provided they appear to be of equal fundamental scientific merit. Part of the burden of decision falls on the scientists themselves. The fact that cancer is better supported right now than some other areas puts a strain on the intellectual and moral integrity of scientists that they must face.

The best basic research can, however, lie dormant unless someone recognizes its possible application to specific problems. The sulfa drugs and penicillin were not immediately applied to the disease problems for which they eventually proved so fruitful. If cancer research resources are used to support basic research of workers whose primary interest is in areas other than cancer, someone must watch the results for possible practical application. This requires that good communication be maintained between different persons by meetings such as this and by scientific publications.

The magnitude of financial support of the three main divisions in cancer, namely, patient care, education, and research shows little correlation with the one, two, seven ratio already mentioned. Perhaps there should be no correlation. The humanitarian problem of case detection, diagnosis, treatment, and care now gets by far the largest portion of the total financial support, almost regardless of the average cost figure used for each of the 300,000 annual cases. Education appears to be under-supported, if to the budgets of lay and graduate physician education are added that portion of each medical school's cost devoted to cancer teaching. Research is now supported better than ever before, but more support could be used and, perhaps, should be allocated if the quantitative evaluation of the problem given here is correct. It is important that the pressure of the immediate humanitarian problem not be permitted to divert the resources that should be spent on the large research problem. Constant re-evaluation of the position in this regard is necessary.

It should be re-emphasized that victory over cancer may not be so difficult to achieve as would appear at first glance. This is most true because conquest is not necessarily an all-or-none phenomenon. Rather, it may come from the summation of numerous small advances. It is not necessary to attack or cure the disease as a whole—that is, the nearly 300,000 cases—but one by one the several dozen types which comprise the whole and, within the types, the cases as individuals. Progress has been unequal in the different types, and, barring discovery of a fundamental defect in cancer

cells and methods for returning this process to normal, it will probably so continue.

In summary, it is seen that the cancer problem can be evaluated in quantitative and qualitative terms by numbers, types, and inherent difficulties. The problem can also be evaluated in terms of control, education, and research, and in terms of cure, palliation, and prevention.

There is a challenge and an urgency in this problem imposed upon us from without which we should recognize and accept. The reaction generated within us must not disturb our cerebral processes and efficiency, but it should, when added to that motivation which led us to enter this field, stimulate us to our greatest efforts.

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Antagonism Studies with Several Toxic Purines and Purine Nucleosides in Mouse Tissue Cultures*

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The possibility that the nucleic acid metabolism of cancer cells differs sufficiently from that of normal cells to allow a selective inhibition of cancer cell growth by means of agents that intervene at one stage or another in nucleic acid metabolism has led to numerous recent investigations (5, 7–10, 12–14, 16–19). Among the agents tested have been purine derivatives and analogs.

2,6-Diaminopurine was regarded as the most effective agent against Crocker mouse Sarcoma 180 in tissue culture (5) until 2-chloroadenine was tested (3). 2,6-Diaminopurine was approximately 15–20 times as toxic to Sarcoma 180 cells as to embryonic mouse skin cells *in vitro* (6).

Information concerning the site and mechanism of action of 2,6-diaminopurine was sought by antagonism studies (5), in which other agents were administered together with 2,6-diaminopurine to the cultures. Diaminopurine:adenine inhibition ratios of 16:1 for Sarcoma 180 and 256:1 for embryonic mouse skin cultures were obtained (6). Adenine gave more protection against diaminopurine than any other substances tested, including hypoxanthine, xanthine, guanine, adenosine, crotonoside, muscle or yeast adenylic acids, guanylic acid, glycine, thymine, and folic acid (5).

The studies with 2,6-diaminopurine have now been extended. Similar work has been carried out with certain other purines and purine nucleosides causing damage to Sarcoma 180 cells in culture.

MATERIALS AND METHODS

The technic employed was much the same as that described in previous papers (3, 6). Roller tube tissue cultures were treated, after a day of growth, with various purines and purine derivatives. Instead of adding only one agent, as in screening tests (3), now two were added. Hence, 0.2 ml. of the fluid medium was removed to allow replacement of two volumes of 0.1 ml. saline containing the two agents. Observations were made as before. It was found desirable to hold the concentration of one agent constant at a level just sufficient to cause definite damage and to vary the concentration of the substance being tested for protection. Companion tests of the toxicity of each agent alone in the concentrations used were also made. Resultant cellular damage is indicated in the tables by signs of 3+ for extensive, 2 for moderate, 1 for slight, and a 0 for no damage.

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RESULTS

Four purines in addition to those previously reported (6) were tested for protection against 2,6-diaminopurine lactate[1] (Table 1). None was found

TABLE 1

EFFECTS OF VARIOUS PURINES ON THE TOXICITY OF 2,6-DIAMINOPURINE LACTATE

Concentration (μM/ml)		Damage to cultured cells	
Diamino-purine	Other purine	Embryo skin	Sarcoma 180
	6-methylaminopurine		
0.1			+++
0.1	0.5		++ to +++
0.1	0.1		+++
	0.5		+
	0.1		0
	2-aminopurine		
1.6		+ to ++	
1.6	3.3	++	
1.6	1.6	++	
1.6	0.4	+ to ++	
	3.3	0	
0.1			++
0.1	3.3		++
0.1	0.4		++
0.1	0.1		++
	3.3		+
	6-acetamidopurine		
2.0		++	
2.0	2.0	0	
2.0	0.5	+	
2.0	0.125	+ to ++	
	2.0	+	
0.1			+++
0.1	0.4		++
0.1	0.1		++
0.1	0.025		+++
	0.4		0
	2,6-diacetamidopurine		
1.5		++	
1.5	4.0	++	
1.5	1.0	++	
0.1			++
0.1	4.0		++ to +++
0.1	1.0		+++
	4.0	+	++
	1.0	0	0

[1] These compounds were kindly provided by Miss Gertrude B. Elion and Dr. George H. Hitchings of the Wellcome Research Laboratories.

to have an efficacy near that of adenine sulfate[2] (6). The protection given to both embryo skin cells and Sarcoma 180 cells by 6-acetamidopurine[3] was equivalent to that given by $\frac{1}{64}$th its concentration of adenine sulfate. No blocking was observed with 2,6-diacetamidopurine,[3] 6-methylaminopurine,[1] or 2-aminopurine.[1]

The toxic effects of 2-chloroadenine[3] on Sarcoma 180 cells were readily blocked by equimolar concentrations of adenine sulfate (Table 2; Figs. 1, 2).

TABLE 2

EFFECTS OF VARIOUS PURINES ON THE TOXICITY OF 2-CHLOROADENINE

CONCENTRATION (μM/ML) 2-Chloroadenine	Other purine	DAMAGE TO CULTURED CELLS Sarcoma 180
adenine sulfate		
0.1		+++
0.1	1.0	0
0.1	0.2	0
0.1	0.1	0
0.1	0.025	++
0.1	0.006	+++
0.05		++ to +++
0.05	0.1	+
0.05	0.05	+
0.05	0.025	+ to ++
0.05	0.0125	++
0.05	0.0063	++
2,6-diaminopurine lactate		
0.05		++
0.05	0.01	++
	0.01	0
2,8-dichloroadenine		
0.05		++ to +++
0.05	0.1	++ to +++
0.05	0.05	++ to +++
	0.1	0 to +

No protection against 2-chloroadenine was observed with the few concentrations of 2,8-dichloroadenine[3] and 2,6-diaminopurine lactate tested.

Adenine failed to affect the toxicity of two other selectively sarcoma-damaging purines. The moderate damage to fetal skin cultures caused by 3.0 μM/ml of 8,8′-ethylenediaminoditheophylline[2] was not influenced by adenine sulfate at 3.0, 0.75, and 0.18 μM/ml; nor was the moderate damage to Sarcoma 180 cells caused by 2.0 μM/ml of this substituted theophylline affected by 2.0, 0.5, and 0.125 μM/ml of adenine sulfate. 2-Methyladenine[3] at 1.0 μM/ml caused moderate damage to Sarcoma 180 cells, which was not affected by adenine sulfate at 2.0, 1.0, or 0.25 μM/ml.

Of five nucleic acid preparations tested for ability to block damage from 2,6-diaminopurine (Table 3), two preparations gave protection. These two were found to contain free adenine to the extent of several per cent,[4] sufficient to explain the observed blocking. Sarcoma 180 desoxyribonucleic acid[5] and dialyzed fractions of yeast polynucleotide residue[6] after ribonuclease treatment gave no protection.

Table 4 summarizes experiments on the effects of adenosine triphosphate and cozymase on the toxicity of 2,6-diaminopurine lactate and 2-chloroadenine in Sarcoma 180 cultures. Both coenzyme I and tetrasodium adenosine triphosphate preparations damaged Sarcoma 180 cells at 1.0 μM/ml and above, and this toxicity appeared to overlie the

TABLE 3

EFFECTS OF NUCLEIC ACID PREPARATIONS ON THE TOXICITY OF 2,6-DIAMINOPURINE LACTATE

DIAMINOPURINE μM/ml	NUCLEIC ACID μg/ml	DAMAGE TO CULTURED CELLS Sarcoma 180
yeast polynucleotide (contained free adenine)		
0.1		++
0.1	600	+
0.1	150	+
0.1	38	++
	600	0 to +
yeast polynucleotide, dialyzed residues after ribonuclease		
0.1		++
0.1	600	++
0.1	150	++
0.1	38	++
	600	0 or +
desoxyribonucleic acid from sperm (contained free adenine)		
0.1		+++
0.1	1200	+
0.1	300	+
0.1	75	++ to +++
	1200	0
desoxyribonucleic acid from mouse Sarcoma 180		
0.1		+++
0.1	600	+++
0.1	150	+++
0.1	38	+++

blocked toxicity of the purines. A molar concentration of about 10 times that of diaminopurine was needed so that the ATP preparation protect.

[2] These compounds were kindly provided by Merck & Co.

[3] These compounds were kindly provided by Dr. G. B. Brown and Dr. John Davoll of the Bio-organic Chemistry Section of the Sloan-Kettering Institute.

[4] Analyses with paper chromatography were kindly carried out by Dr. Aaron Bendich of this Institute.

[5] This material was kindly supplied by Dr. G. Ceriotti while with the Division of Experimental Chemotherapy of the Sloan-Kettering Institute.

[6] Dr. G. B. Brown of the Bio-organic Chemistry Section of the Sloan-Kettering Institute kindly provided yeast polynucleotide and two samples of dialyzed yeast polynucleotide residue after ribonuclease treatment.

Relatively more was required of both the ATP and coenzyme I preparations to protect against 2-chloroadenine than against 2,6-dimaniopurine. The coenzyme I preparation was about 16 times more effective against 2,6-diaminopurine than against 2-chloroadenine. This is also the relative effectiveness of adenine sulfate in blocking the two toxic purines. Paper chromatography demonstrated the presence of 2.2 per cent free adenine in the coenzyme I preparation,[4] a quantity large

TABLE 4

EFFECTS OF ADENOSINE TRIPHOSPHATE AND COENZYME I ("90 PER CENT PURITY") ON TOXICITY OF 2,6-DIAMINOPURINE AND 2-CHLOROADENINE

Concentration (μM/ml)				Damage to cultured cells
Diamino-purine	2-Chloro-adenine	ATP	Co I	Sarcoma 180
		10.0		++
		5.0		+ to ++
		1.0		0 to +
0.1				+++
0.1		10.0		++
0.1		5.0		+ to ++
0.1		1.0		+
0.1		0.1		+++
	0.05			++ to +++
	0.05	10.0		++
	0.05	5.0		++
	0.05	1.0		+++
			1.2	+ to ++
			0.6	+
			0.075	+
			0.019	0 to +
0.1				++
0.1			0.6	+
0.1			0.075	+
0.1			0.019	++
	0.05			++ to +++
	0.05		1.2	+ to ++
	0.05		0.6	+
	0.05		0.075	++

enough to account for most if not all of the protective effect of the cozymase preparation. If coenzyme I did of itself exert any protection against 2,6-diaminopurine or 2-chloroadenine, it did so, like the adenosine triphosphate preparation, at a considerably lower efficiency than adenine.

Attempts to block toxic purine nucleosides made up the rest of the experimental work. Table 5 summarizes adenine sulfate blocking of 2,6-diamino-9-β-D-ribofuranosylpurine (2-aminoadenosine).[7] Adenine did not effectively block 4.0 μM/ml of 2-aminoadenosine, but 2.0 μM/ml of 2-aminoadenosine were reduced in toxicity by 0.5 μM of adenine sulfate.

Adenosine at 1.0 or more μM/ml did successfully block the toxic effects of 4.0 μM/ml 2-aminoadenosine (Table 6). A reduction in toxicity of the same relative extent for both embryo skin and Sarcoma 180 cells was brought about by 0.5 μM/ml adenosine when the 2-aminoadenosine concentration was 2.0 μM/ml. In contrast, guanosine, which is itself at moderate concentrations innocuous in tissue cultures (3), appeared to increase the extent of damage caused by 2-aminoadenosine. These effects are illustrated in Figures 3–5.

Adenosine and adenine sulfate were less effective in blocking 2-chloroadenosine[7] (Table 7). Embryonic skin cells were slightly protected against

TABLE 5

EFFECT OF ADENINE SULFATE ON TOXICITY OF 2-AMINOADENOSINE

Concentration (μM/ml)		Damage to cultured cells	
2-Amino-adenosine	Adenine sulfate	Embryo skin	Sarcoma 180
4.0		+++	+++
4.0	2.0	+ to +++	+++
4.0	1.0	++ to +++	+++
4.0	0.5	++ to +++	+++
4.0	0.125	++ to +++	+++
2.0		+ to ++	++ to +++
2.0	2.0	+ to ++	+ to ++
2.0	1.0	+ to ++	+
2.0	0.5	+	+
2.0	0.125	+	+ to ++

TABLE 6

EFFECTS OF ADENOSINE AND GUANOSINE ON THE TOXICITY OF 2-AMINOADENOSINE

Concentration (μM/ml)			Damage to cultured cells	
2-Amino-adenosine	Adeno-sine	Guano-sine	Embryo skin	Sarcoma 180
4.0			+++	+++
4.0	4.0		0 to +	0 to +
4.0	1.0		0 to +	+
4.0	0.25		+++	++
4.0		4.0	+++	+++
4.0		1.0	+++	+++
4.0		0.25	+++	+++
2.0			+ to ++	++ to +++
2.0	2.0		0 to +	0 to +
2.0	0.5		0 to +	0 to +
2.0	0.125		+	+ to ++
2.0		2.0	++	+++
2.0		0.5	++	+++
2.0		0.125	++	++ to +++

2-chloroadenosine by equimolar concentrations of adenosine, but similar concentrations of adenine sulfate were without protective effect. On the contrary, Sarcoma 180 cells were protected better by adenine sulfate than by adenosine. The resultant reduction in toxicity of 2-chloroadenosine for Sarcoma 180 cells was but slight in extent.

Guanine hydrochloride at 0.5, 1.0, and 2.0 μM/ml did not block the toxic effects of 2.0 μM/ml of 2-aminoadenosine with either tissue. Nor was the toxicity of 0.2 μM/ml of 2-chloroadenosine influ-

[7] These compounds were kindly supplied by Dr. G. B. Brown, Dr. John Davoll, and Mr. Bertram Lowy of the Bioorganic Chemistry Section of the Sloan-Kettering Institute.

enced by guanine at concentrations of 0.05–0.8 μM/ml.

Adenosine at equimolar or greater concentration was capable of blocking the toxic effects of 9-β-D-xylofuranosyladenine[7] (an impure preparation) toward both embryo skin and Sarcoma 180 cells. Adenosine at 2.0, 0.5, and 0.125 μM/ml did not block the extensive damage from crotonoside[8] at 2.0 μM/ml in embryo skin or Sarcoma 180 cultures. Nor did adenosine block the toxic effects of 2-methyladenosine[7] as observed at 46 hours of exposure. Instead, the slight damage to Sarcoma 180 cells from 1.0 μM/ml of 2-methyladenosine was increased to moderate or extensive damage by the same amount of adenosine.

TABLE 7

EFFECTS OF ADENOSINE AND ADENINE SULFATE ON THE TOXICITY OF 2-CHLOROADENOSINE

Concentration (μM/ml)			Damage to cultured cells			
			Embryo skin		Sarcoma 180	
2-Chloro-adenosine	Adenosine	Adenine	24 hours	48 hours	24 hours	48 hours
0.4			+	++		
0.4	1.6		0 to +	+ to ++		
0.4	0.4		0 to +	++		
0.4	0.1		+ to ++	++		
	1.6		+	+		
0.4		1.6	+	++		
0.4		0.4	+	++		
0.4		0.1	+	++		
		1.6	+	++		
0.2			+ to ++		++ to +++	
0.2	0.4		0 to +		++	
0.2	0.2		+		++ to +++	
0.2	0.05		+		++ to +++	
	0.4		0		0	
0.1					++	+++
0.1	0.4				+ to ++	+++
0.1	0.1				++	+++
0.1	0.025				++	+++
	0.4				0	0 to +
0.1		0.4			+	++
0.1		0.1			+	++ to +++
0.1		0.025			+ to ++	+++
		0.4			0	0 to +

DISCUSSION

It had been previously discovered that, of a series of biochemicals ranging in complexity from purines to adenylic acid and folic acid, only adenine gave good protection to mouse tissue cultures against 2,6-diaminopurine (6). An additional number of purines may now be added to the list of essentially ineffective blocking agents. Substances of greater structural complexity have also proved ineffective. Much of the protection against 2,6-diaminopurine lactate that was afforded by several preparations of nucleic acids, adenosine triphosphate, and coenzyme I could be attributed to free adenine contained in the preparations. The interpretation of 2,6-diaminopurine toxicity as primarily an anti-adenine effect is still on firm ground. However, there is no resolution of the two possibilities, that the antiadenine effect is strictly an antimetabolite effect or is the expression of a disturbed adenine-guanine balance in nucleic acids (2). These two possibilities need not be mutually exclusive.

2-Chloroadenine may also be considered an anti-adenine agent, but whether its effect is primarily anti-adenine has not been determined.

The inhibition ratios for 2-chloroadenine:adenine and 2,6-diaminopurine:adenine were different. This ratio was determined from the concentration of adenine sulfate needed to reduce the tissue damage to slight, when the concentration of the antimetabolite alone was just sufficient to cause extensive damage (6). The 2-chloroadenine:adenine inhibition ratio for Sarcoma 180 cells was about 1:1, in contrast to the 2,6-diaminopurine:adenine ratio of 16:1. For protection of Sarcoma 180 against the more toxic of the two antimetabolites, a greater amount of adenine was required.

Since saturated solutions of 2-chloroadenine were hardly toxic to embryonic skin cells, an inhibition ratio with adenine could not be determined. If the 2-chloroadenine:adenine inhibition ratio for embryonic mouse skin cells were very high, e.g., in excess of the 256:1 of the diaminopurine:adenine ratio (6), then 2-chloroadenine could not cause more than slight damage to embryonic skin cells *in vitro*, if the medium or the

[8] Crotonoside was kindly supplied by Dr. G. B. Brown and Dr. Aaron Bendich of the Bio-organic Chemistry Section of the Sloan-Kettering Institute.

initial explants contained a minimal quantity of free adenine.

The failure of adenine sulfate to block 2-methyladenine and 8,8′-ethylenediaminoditheophylline may have resulted in part from inhibition ratios so low that toxic quantities of adenine would be needed to block toxic amounts of the other two. Antagonism would not then be detectable.

Possibly some purines might damage cells in other ways than by anti-adenine action. This would be suggested by the morphological effect of the theophylline derivative. It caused the cells to be large, rounded, and highly granular instead of shrunken and disintegrated as they become when treated with 2,6-diaminopurine. Kidder and Dewey (11), finding that only about one-half the inhibitory effect of methylxanthines on Tetrahymena could be prevented with purines, suggested that methylxanthines inhibited some nonpurine as well as purine enzyme systems.

Antagonism studies with purine nucleosides suggest more than one mechanism of action for the toxic nucleosides studied. The obvious hypothesis to test, that these nucleosides exerted an anti-adenosine effect, was corroborated to some extent for several of the nucleosides but not for others.

The toxic effects of 2-aminoadenosine were blocked by adenosine, but not by guanosine. The cytological effects of guanosine at higher concentrations tended toward those caused by a combination of adenosine and 2-aminoadenosine, but not by adenosine alone (4). These facts are not at variance with the adenine-guanine disturbance hypothesis (2), with 2,6-diaminopurine recognized as a precursor of nucleic acid guanine (1), perhaps proceeding through preliminary conjugation with ribose (15).

Adenosine gave a little protection against the toxic effects of 2-chloroadenosine and 9-β-D-xylofuranosyladenine. However, it was ineffective against crotonoside and against 2-methyladenosine, just as adenine failed to block 2-methyladenine.

These observations indicate that the toxicity of the purine nucleosides to cultivated mouse tissues cannot be accounted for solely on the basis of adenosine antagonism and that there may be several different mechanisms of action.

Because of the possible hydrolysis of purine nucleosides at incubation temperatures, the likelihood that the nucleoside toxicity observed was actually the toxicity of purines released from the nucleosides must be considered (3). The present antagonism studies provide evidence against this possibility. Both 2-aminoadenosine and 2-chloroadenosine were blocked more effectively by adenosine than by adenine. 2,6-Diaminopurine, among the purine moieties of the toxic nucleosides, was blocked by adenine but not by adenosine (6). The 2-aminoadenosine : adenosine inhibition ratios were the same for Sarcoma 180 and embryonic skin cells, but the 2,6-diaminopurine : adenine inhibition ratios were markedly different for cells from the two sources. Contributory evidence against the possibility is seen in the greater toxicity of crotonoside and of xylofuranosyladenine than of their free purine moieties and in the importance of the sugar and its configuration (3). Hence, it would appear that the whole nucleoside molecule was involved in the expressions of toxicity considered here.

SUMMARY

1. Attempts were made to block the toxic effects of a number of purines and purine nucleosides in roller-tube mouse tissue cultures.

2. Four additional purines failed by far to match adenine sulfate in ability to block 2,6-diaminopurine lactate toxicity.

3. Such protection against 2,6-diaminopurine lactate as was afforded by five preparations of nucleic acids, by adenosine triphosphate, and by coenzyme I was accounted for, at least in part, on the basis of free adenine that contaminated the preparations.

4. Adenine sulfate protected Sarcoma 180 cells against 2-chloroadenine, with an inhibition ratio of 1 : 1.

5. Adenine sulfate failed to block 2-methyladenine and 8,8′-ethylenediaminoditheophylline.

6. 2-Aminoadenosine was blocked by adenosine and almost as well by adenine. Guanine did not block, and guanosine increased the damage.

7. 2-Chloroadenosine was partly blocked by adenosine. There was a slight effect from adenine but none from guanine.

8. Adenosine gave slight protection against 9-β-D-xylofuranosyladenine but none against crotonoside or 2-methyladenosine.

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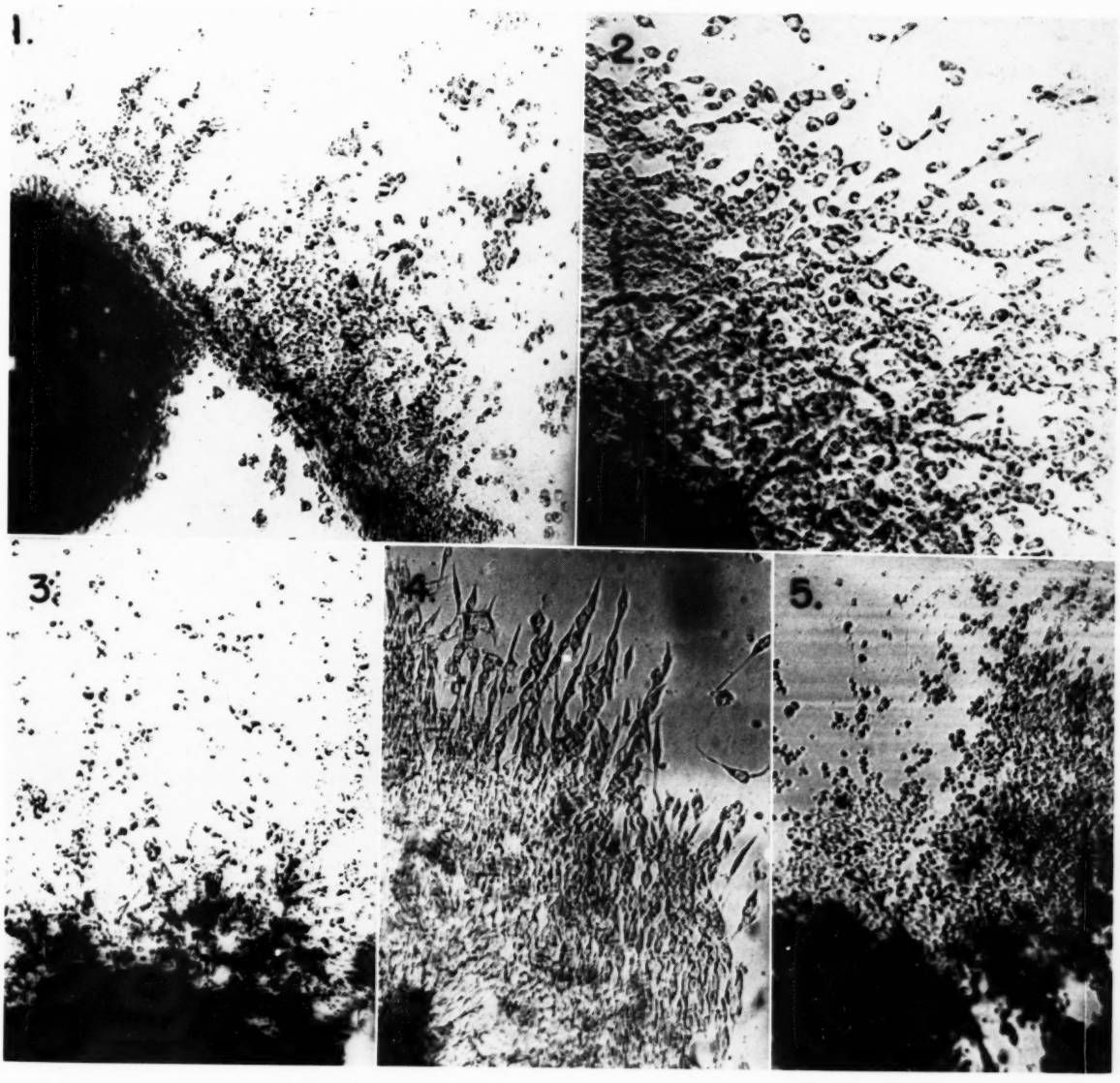
Fig. 1.—Sarcoma 180 culture showing extensive damage after 24 hours' exposure of 0.1 μM/ml of 2-chloroadenine. Mag. ×75.

Fig. 2.—Sarcoma 180 culture in good condition after 24 hours' exposure to 0.1 μM/ml of 2-chloroadenine plus 0.2 μM/ml of adenine sulfate. Mag. ×75.

Fig. 3.—Sarcoma 180 culture showing moderate to extensive damage after 24 hours' exposure to 2.0 μM/ml of 2-aminoadenosine. Some cells still have extended processes. Mag. ×75.

Fig. 4.—Sarcoma 180 culture in good condition after 24 hours' exposure to 2.0 μM/ml of 2-aminoadenosine plus 2.0 μM/ml of adenosine. Mag. ×75.

Fig. 5.—Sarcoma 180 culture showing extensive damage after 24 hours' exposure to 2.0 μM/ml of 2-aminoadenosine plus 2.0 μM/ml of guanosine. Mag. ×75.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Observations on Carcinogenesis by 4-Dimethylaminoazobenzene*

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The experiments described herein resulted from the continuation of a line of studies reported earlier (8, 9). To avoid a multiplicity of papers, a variety of related and unrelated factors has been grouped for consideration, and, for the sake of clarity, the various factors will be discussed in separate units of this publication. Since the methods employed were uniform, a single description will suffice.

GENERAL METHOD

Purified diets were prepared according to the formulas given in Tables 1–4 and 6 and were kept in a refrigerator. Except when the number of rats on a diet was small, the diets were made in batches of 5 kg., and additional batches were usually made at intervals of 3 or 4 weeks. The 4-dimethylaminoazobenzene (DAB), obtained from the Eastman Kodak Company, was dissolved in oil at a concentration of 2 per cent with the aid of gentle heating before incorporation into the diet. Cottonseed oil was usually used, but in some diets it was replaced with olive oil or corn oil. The carcinogen content of one-third of the diets was 0.6 per cent, but in the remainder it was 0.09 per cent. The higher level was originally used, and the lower level was tried with the hope of reducing the mortality rate in the first weeks of each experiment. Since this hope was not fully realized and since it seemed that we had incited a somewhat more rapid carcinogenesis with the higher concentration, we ultimately returned to it. Except in one experiment, the rats were of the Harlan strain, obtained from a local dealer and derived from the Wistar strain. The Harlan strain was used in our earlier studies. The rats were preponderantly males, and, at the beginning of treatment, usually weighed 90–120 gm. They were kept in groups of four or less per cage in an air-conditioned room at a temperature of 76° to 80°F. The usual number started on a diet was 24, but, when many deaths occurred soon after a group was started, replacements were made. To prevent accumulation of old food, the food cups were allowed to become empty over the week-ends, but at other times they were filled daily if necessary. The rats were kept on the carcinogen-containing

TABLE 1

COMPOSITION OF DIETS 43, 44, 57, AND 58

	Diet 42	Diet 44	Diet 57	Diet 58
Casein, vitamin-free, Labco	100.0	100.0	100.0	100.0
Corn starch	780.0	780.0	765.0	765.0
Cottonseed oil	80.0	80.0	95.0	95.0
McCollum's salt mixture no. 185*	40.0	40.0	40.0	40.0
Vitamin D concentrate in oil, 400,000 IU per gm.	0.005	0.005	0.005	0.005
Carotene	0.01	0.01	0.01	0.01
Calcium pantothenate	0.006	0.006	0.006	0.006
Thiamine	0.005	0.005	0.005	0.005
α-Tocopherol (as distilled natural tocopherols)	0.01	0.01	0.01	0.01
Niacin	0.03	0.03	0.03	0.03
Pyridoxine	0.003	0.003	0.003	0.003
Choline chloride	0.03	0.03	0.03	0.03
Riboflavin	0.002	0.002	0.002	0.002
4-Dimethylaminoazobenzene	0.6	0.6	0.9	0.9
Folic acid	0.0002			0.0019

* Modified by addition of 0.4 gm. of KI, 0.72 gm. of $CuSO_4$, and 1.0 gm. of $MnCl_2$ to 5916.6 gm. of salts mixed according to McCollum's formula.

Since items 4 to 12 (salt mixture to choline chloride) were constant except as noted below, they will not be repeated in Tables 2–5. The pyridoxine level in diet 68 was 2.4 mg/kg. α-Tocopherol was omitted from diets that contained corn oil. The choline chloride level in diets 51, 52, 53, 54, 81, and 82 was increased to 1 gm/kg, and the niacin level was reduced to 0.01 gm/kg.

* Most of this material was presented at the 1948 and 1949 meetings of the American Association for Cancer Research.

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diet throughout life. After 3 or 4 months on the diets, the animals were palpated at weekly intervals, and, when it was deemed certain that a liver tumor was present, the animal was killed and the liver preserved for histologic examination. In calculating tumor incidence, shown in the charts, the number of those rats living on a diet at the time the first tumor was discovered and of those subsequently examined at necropsy has been used. This number is referred to as the effective total in Table 7, which also summarizes for all diets the number of rats used, the number that developed tumors, and the minimum latent period. Other data may be derived from the charts.

TABLE 2

COMPOSITION OF DIETS 59, 60, 61, 63, 64, 65, 73, 74, AND 75

	Diet 59	Diet 60	Diet 61
Casein, vitamin-free, Labco	100.0	100.0	100.0
Corn starch	810.0	760.0	660.0
Cottonseed oil	50.0	100.0	200.0
Items 4–12 in Table 2 as in Table 1 in all these diets			
Riboflavin	0.002	0.002	0.002
4-Dimethylaminoazobenzene	0.9	0.9	0.9

Diets 63, 64, and 65 corresponded with diets 59, 60, and 61, respectively, differing only in that cottonseed oil was replaced by corn oil, and α-tocopherol was not added to the diets.

Diets 73, 74, and 75 differed from diets 59, 60, and 61, respectively, only in containing olive oil instead of cottonseed oil.

TABLE 3

COMPOSITION OF DIETS 76, 77, AND 78

	Diet 76*	Diet 77	Diet 78
Casein, vitamin-free, Labco	100.0	300.0	100.0
Corn starch	760.0	560.0	760.0
Corn oil	100.0	100.0	100.0
Items 4–12 in Table 3 as in Table 1 in all three diets†			
Riboflavin	0.002	0.002	0.1
4-Dimethylaminoazobenzene	0.9	0.9	0.9

* Diet 76 is the same as 64.

† Except that α-tocopherol was omitted.

TABLE 4

PREPARATION OF VARIOUS LIVER FRACTIONS

Raw ground liver is extracted in water at 85° C.

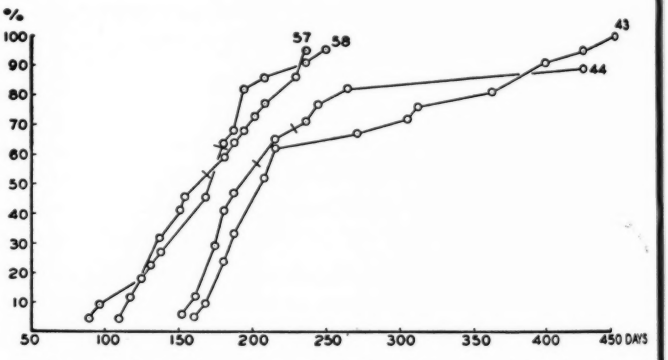
- Filtrate is concentrated *in vacuo* and ethanol is added to a concentration of 70 per cent
  - Filtrate is concentrated *in vacuo* and ethanol is added to a concentration 90 per cent
    - Filtrate (used in diet 48)
    - Precipitate (used in diet 45) Liver Extract (Lilly)
  - Precipitate (used in diet 47) Liver Extract No. 55
- Residue (used in diet 46)

SPECIFIC METHODS AND RESULTS

Folic Acid

The reports of Leuchtenberger and associates (14, 15) that folic acid caused inhibition of the growth of Sarcoma 180 and regression of spontaneous breast carcinoma in mice suggested a study of the effect of this substance upon DAB carcinogenesis. Accordingly, diets 43 and 44 were prepared, 43 containing folic acid at the level of 0.2 mg/kg and 44 being the control. The azo dye content of these diets was 0.06 per cent. The experiment was subsequently repeated with diets 57 and 58, containing 0.09 per cent of DAB, for by this time the decision had been made to return to the higher concentration of dye. The folic acid content was also increased, diet 58 containing 9.5 times as much as diet 43.

Results.—Folic acid had no effect upon the incidence or severity of hepatic cirrhosis, and, as may be seen in Chart 1, there was no significant difference between the rate of tumor development in animals getting folic acid (diets 43 and 58) and that in the controls. Possibly the rats on diets 43 and 44 developed tumors more slowly than those on diets 57 and 58 because of having had a suboptimal daily intake of carcinogen.



Cumulative tumor percentage is plotted against time in days. Death of a tumor-free rat is shown by a short line perpendicular to the curve of cumulative tumor percentage.

CHART 1.—Folic acid and control diets. Diets 43 and 58 contained folic acid.

The Strain of Rat

The possibility that our rats and those used by others might differ in susceptibility to DAB and that, as a consequence, we might get results differing from those of others suggested the following experiment. Five strains of rats—Harlan, Sprague-Dawley, Wistar, Long-Evans, and Carworth Farms—were fed diet 57, which had been found to give fairly rapid tumor development.

Results.—The results are shown graphically in Chart 2. The rate of tumor development in the Harlan and Sprague-Dawley strains was the same. The rate in the Long-Evans strain was also identical until the incidence had reached 40 per cent, when it began to decelerate. However, this may be fortuitous, for the effective total number was lower in this group than in the others. Tumor development in the Wistar strain lagged 4–6 weeks behind that in the Harlan strain. Tumors developed much more slowly in the Carworth Farms rats than in the rats of other strains. These animals were approximately 2 months older than the others when the experiment was begun, and many died during the latent period of tumor development. Since the Carworth Farms strain was derived from the Wistar strain and is generally considered to be very similar to its parent strain, this result is not what would be expected, and its validity is subject to question. Sugiura and Rhoads (24) found

that Sherman and Wistar rats were more susceptible to the carcinogenic action of DAB than were Evans rats. Our results are not in accord with this finding. Although there appears to be prima facie evidence that strains of rats differ in susceptibility to DAB, the question needs further study before this can be said to be true.

COTTONSEED, CORN, AND OLIVE OILS

The possibility that the kind or amount of dietary fat might influence DAB carcinogenesis has received some attention (7, 12, 13, 17, 18, 23) but for the most part has been ignored. Although the fat content of the diets employed by different workers has varied greatly and different types of fats

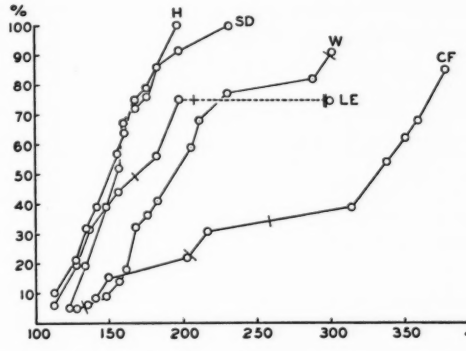


CHART 2.—Different strains of rats. *H*=Harlan strain; *SD*=Sprague-Dawley strain; *LE*=Long-Evans strain; *W*=Wistar strain; *CF*=Carworth Farms strain.

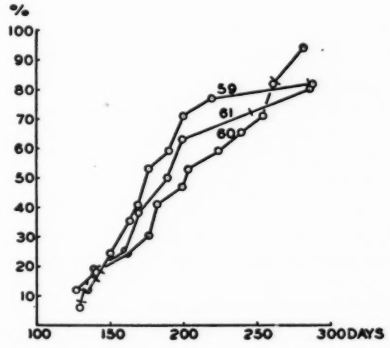


CHART 3.—Cottonseed oil. Diet 59=5 per cent cottonseed oil; diet 60=10 per cent cottonseed oil; diet 61=20 per cent cottonseed oil.

have been used, because of variation in other constituents also, few valid comparisons can be made, and only limited conclusions may be drawn. An additional complication has been introduced by the observation of Czaczkes and Guggenheim (2) that rats fed a diet low in fat required less riboflavin to maintain their stores of this vitamin in the liver than did those on a diet containing 10 per cent of olive oil, and that those on a high fat diet required more riboflavin. This was attributed to depression by high fat diet of intestinal synthesis of riboflavin. The effect of other dietary constituents upon this synthesis should also always be kept in mind.

Since there were no published data on a comparative study in one laboratory of the effect of various concentrations of different oils, it was thought that such an investigation should be carried out, and, since corn, cottonseed, and olive oils had been used commonly as carriers for DAB, diets containing one of these oils in concentrations of 5, 10, and 20 per cent were prepared. Details of the composition of the diets are given in Table 2. An additional diet containing 40 per cent of cottonseed oil was fabricated, but, since much of the oil separated out, the concentration of oil was reduced to 30 per cent, and Ruffex to the extent of 5 per cent was added. This made a gummy mass, of the consistency of peanut butter. Although the diet was unsatisfactory, its administration was continued,

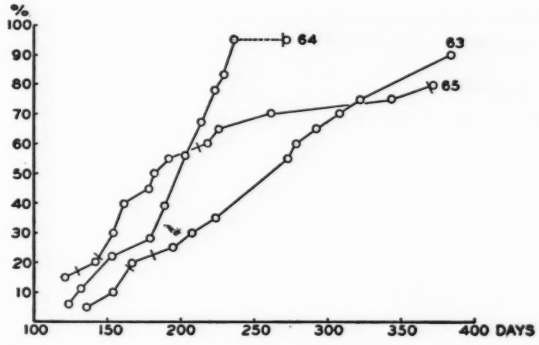


CHART 4.—Corn oil. Diet 63=5 per cent corn oil; diet 64=10 per cent corn oil; diet 65=20 per cent corn oil.

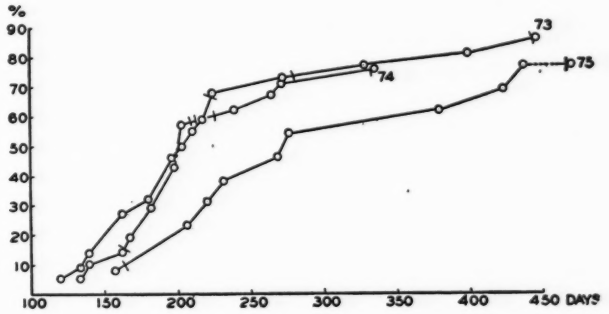


CHART 5.—Olive oil. Diet 73=5 per cent olive oil; diet 74=10 per cent olive oil; diet 75=20 per cent olive oil.

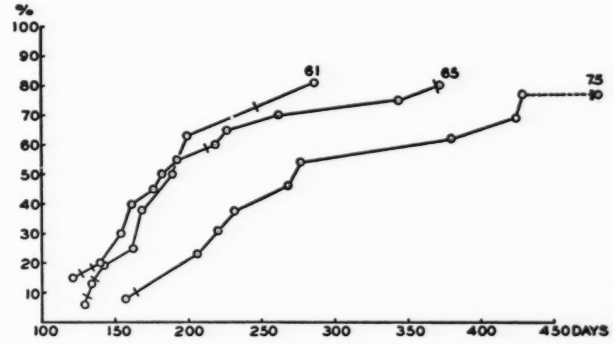


CHART 6.—Diets containing 20 per cent of oil. Diet 61=cottonseed oil; diet 65=corn oil; diet 75=olive oil.

and, as compared to diets containing less oil, it appeared to give slight protection. Since there are several possible explanations, the result is not considered significant. The original intention to prepare diets containing the same concentration of the other oils was forsaken early.

Results.—There was no difference in the rate of tumor development on diets 59, 60, and 61 containing 5, 10, and 20 per cent of cottonseed oil (Chart 3), but tumors developed more slowly in rats on a diet containing 5 per cent of corn oil

(No. 63) than in rats on diets containing 10 or 20 per cent of corn oil (Nos. 64 and 65), as is shown in Chart 4. Kline and associates (12) had previously found that a diet containing 20 per cent of corn oil gave a higher tumor incidence than one containing 5 per cent, and confirmatory results were obtained by Clayton and Baumann (1). Tumor development in rats on diet 75, containing 20 per cent of olive oil, was slower than in those on diets 73 and 74, containing 5 and 10 per cent of olive oil (Chart 5). In graphs of tumor development for diets containing the same concentration of different oils there was no difference between the oils at a level of 10 per cent, but the 20 per cent olive oil diet showed the same degree of tumor retardation (Chart 6) as it did when compared to the other olive oil-

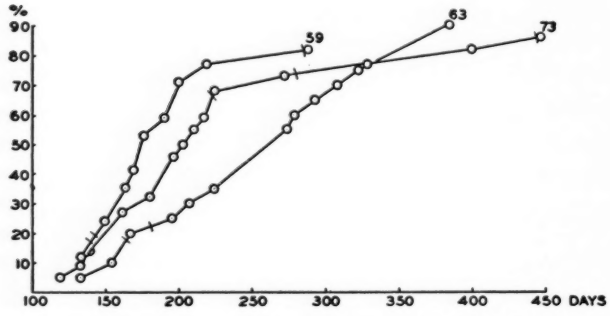


CHART 7.—Diets containing 5 per cent of oil. Diet 59 = cottonseed oil; diet 63 = corn oil; diet 73 = olive oil.

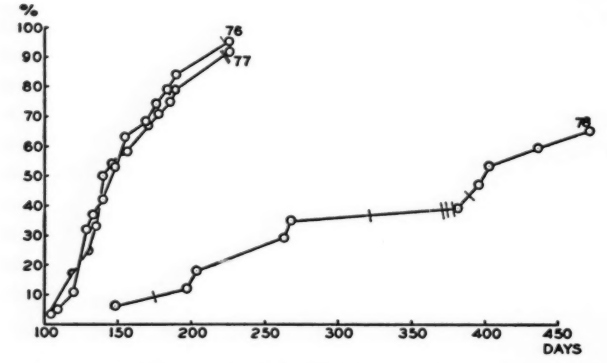


CHART 8.—Riboflavin. Diet 76 = low casein, low riboflavin; diet 77 = high casein, low riboflavin; diet 78 = low casein, high riboflavin.

containing diets, and tumors developed as rapidly with the 20 per cent cottonseed oil diet as with the 20 per cent corn oil diet. Of the 5 per cent oil diets, the one containing corn oil showed the slowest tumor development (Chart 7). The findings were in part confirmatory of those of the Wisconsin workers (1, 12), but not all results were what might be expected.

RIBOFLAVIN

Kensler and associates (11) found that a combination of riboflavin and casein gave much more protection against carcinogenesis by DAB than did either alone, and the early findings of Miller and associates (19) confirmed the impression that protection by riboflavin depended upon the presence of a certain amount of protein. Our data too seemed to verify this interpretation (8). The observations of Miner and associates (20) that riboflavin alone was protective when given in sufficient amount was extended by Griffin and Baumann (4), who gave an explanation for the discrepancy between these and earlier results. We had long planned to make a comparison of the effects of riboflavin and casein in the long-term manner used in our experiments, and, although the last mentioned publication made the study appreciably less essential, it still seemed worthy of pursuit; and diets 76, 77, and 78 were prepared as shown in Table 3. Diet 76 contained 10 per cent of casein and 2 mg riboflavin/kg; diet 77 contained 30 per cent of casein and 2 mg riboflavin/kg; and diet 78 contained 10 per cent casein and 100 mg riboflavin/kg.

Results.—Chart 8 shows that there was no difference in tumor development in rats given diets low in riboflavin and differing in casein content and that tumor development was greatly retarded, but not prevented, on a diet high in riboflavin and low in casein.

LIVER FRACTIONS

It had been known for some time that liver and liver extracts protect against the effects of DAB, but it was not known whether the protective effect was due solely to riboflavin or whether other protective constituents were also present. As a preliminary approach to this problem, diets containing four fractions of liver were prepared. The method of preparation of the fractions is shown in Table 4, and the formulas of the diets are given in Table 5. The various liver fractions in diets 45, 46, 47, and 48 were added in amounts equivalent to the same weight of fresh liver. Sixty-one kg. of fresh liver yielded 4.53 kg. of Liver Extract (Lilly—formerly called Liver Extract No. 343), 8.13 kg. of residue, 1.82 kg. of Extract No. 55, and 924 cc. of filtrate. Assuming the solid fractions to consist almost en-

TABLE 5

COMPOSITION OF DIETS 45, 46, 47, 48, 67, 68, AND 69

	Diet 45	Diet 46	Diet 47	Diet 48	Diet 67	Diet 68	Diet 69
Casein, vitamin-free, Labco	100.0	100.0	100.0	100.0	70.0	100.0	90.0
Corn starch	730.0	700.0	748.0	754.0	810.0	810.0	810.0
Cottonseed oil	100.0	100.0	100.0	100.0			
Items 4–12 in Table 5 as in Table 1 in all seven diets							
Corn oil					50.0	50.0	50.0
Riboflavin*	0.0104	0.0042	0.0032	0.002	0.0072	0.0072	0.0072
Liver Extract (Lilly)	30.0				30.0		10.0
Liver residue		60.0					
Liver Extract No. 55			12.0				
Alcohol soluble filtrate from Liver Extract (Lilly)(see Table 4)				6.0			
4-Dimethylaminoazobenzene	0.6	0.6	0.6	0.6	0.9	0.9	0.9

* Including riboflavin added by the liver fractions. The figure for diet 48 is the amount incorporated in the diet; the filtrate was not assayed and is assumed to have been free of riboflavin.

The pyridoxine levels of diets 45 to 48 are not known, since the extracts and liver residue were not assayed for pyridoxine, but on the basis of assay of another lot of Liver Extract (Lilly), it is probable that diet 45 contained 3.6 mg of pyridoxine/kg. The pyridoxine content of diets 67 and 69 was 3 mg/kg and that of diet 68 was 2.4 mg/kg.

tirely of protein, the quantity of protein added to the diet by these fractions amounted to 3, 6, and 1.2 per cent, respectively, and the quantity of additional riboflavin amounted to 8.4 mg/kg, 2.2 mg/kg, and 1.2 mg/kg, respectively. The diets thus varied appreciably in riboflavin content, which is important, and moderately in protein content, which is probably unimportant. In order to obviate this variation, diets 67, 68, and 69 were prepared with a uniform level of protein and of riboflavin by taking into account beforehand the riboflavin content of the liver extract, the amount of riboflavin in diet 67 provided by the liver extract, of course determining the amounts to be added to diets 68 and 69.

The experiments were impaired by the undesirably large amounts of riboflavin in the diets, but, until we can find a way to extract selectively riboflavin from the fractions, destroy it without injury to other substances, or to antagonize its effects quantitatively, unequivocal results cannot be obtained.

Results.—As is shown by Chart 9, diet 47, containing Extract No. 55, afforded slight protection, as compared to control diet 44; but diet 45, containing Liver Extract (Lilly), was not superior to diet 46, which contained liver residue. Contrary to expectation, tumor development on diet 68 was slower than on liver extract-containing diets 67 and 69 (Chart 10).

The results with diets 44–47 suggest that riboflavin is not the only protective factor present in liver. That there are other protective factors in liver is indicated by the observations of Kensler (10), who found that a highly purified anti-pernicious anemia fraction of liver, which was low in riboflavin, was protective; and by the report of Nelson and associates (22) that liver residue, ostensibly free from riboflavin, used in an appreciably larger amount than in our experiment, gave as good protection as did liver extract. The results with diets 67, 68, and 69 suggest that liver contains a factor that, under proper conditions, favors tumor development. We found this to be true when liver extract constituted 15 per cent of the diet (8, 9), and the biotin intake was thus increased to the level found by du Vigneaud and associates (3) to nullify the action of a protective diet. It is doubtful that the effect noted here could be due to either biotin or pyridoxine.

THIOSALICYLIC ACID AND THIOURACIL

In earlier experiments with DAB we had found that the addition of the amino acids cystine and cysteine to a protective diet gave further protection against tumor development (8). The question arose as to whether or not other compounds containing S=C or HS—C linkages would also prove protective. Thioglycollic and thiosalicylic acids and thiouracil were chosen as suitable substances to try first.

Thioglycollic acid was added to the diet at a level of 1 per cent, and the amount was soon reduced to 0.5 per cent, because the animals would not eat. The reduction made no appreciable difference, and this portion of the study was abandoned.

Thiosalicylic acid.—Thiosalicylic acid was added at a level of 1 per cent (diets 50 and 54) to two types of basal diet. One favored rapid tumor development (No. 44), and the other offered protection against tumor development (No. 53). Details of the composition of the diets are given in Table 6.

Results.—There was a definite retardation of tumor development, as is shown in Chart 11. Tumor development on diet 50 ran steadily 2 months later than on the control diet 44. The effect of thiosalicylic acid was even more striking with the other diet. Tumors appeared slowly with control diet 53, but

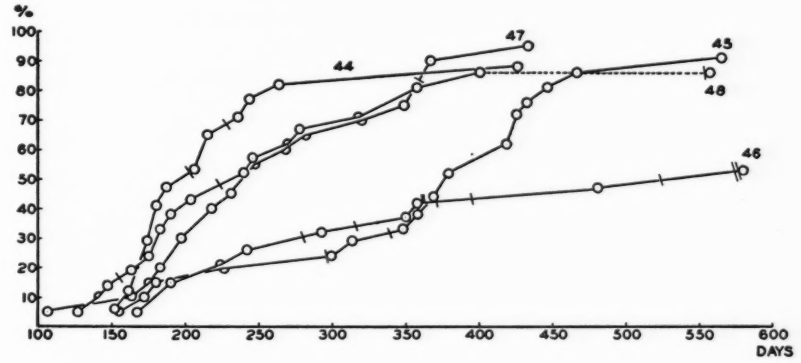


CHART 9.—Liver fractions. Diet 44=control; diet 45=Liver Extract (Lilly); diet 46=liver residue; diet 47=Liver Extract No. 55; diet 48=filtrate from Liver Extract (Lilly).

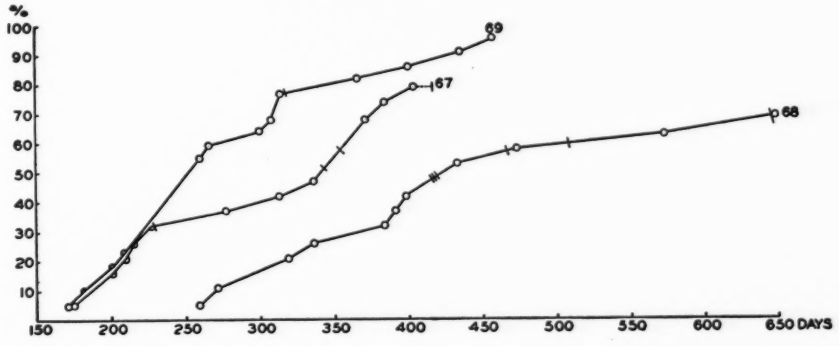


CHART 10.—Liver extract. Diet 67=3 per cent Liver Extract (Lilly) and 7 per cent casein; diet 68=10 per cent casein; diet 69=1 per cent Liver Extract (Lilly) and 9 per cent casein. Riboflavin in all three diets was 7.2 mg/kg.

ultimately ten of the effective total of seventeen rats had developed tumors after a minimum latent period of 256 days. Only one rat fed diet 54 developed a tumor, and this was detected after 422 days; at this time only five rats on diet 54 were still living. Eighteen rats on diet 54 were living when the first tumor was detected in the rats on control diet 53, and seventeen of these died tumor-free after the following intervals on the diet: one after 302 days, four after 358–399 days, eleven after 413–477 days, and the last after 519 days.

Both groups of rats that received thiosalicylic acid weighed a little less than the controls. The rats on diet 50 maintained a constant weight throughout the experiment. For 4 months the control rats weighed about the same as the rats on diet 50, but then began to gain; and after 3 more months averaged about 30 gm. heavier. The rats on diet 54 gained weight steadily, but at a somewhat slower rate than the controls.

Thiosalicylic acid had no effect upon the severity or incidence of cirrhosis. All rats on the 10 per cent protein diets 44 and 50 had cirrhosis. Two of the effective total of ten rats on diet 53 had slight cirrhosis, and the remainder were free of cirrhosis. No rat on diet 54 developed gross cirrhosis, and sections were made only of the liver of the tumor-bearing rat.

increased 50 per cent, for it had been decided that, thereafter, the azo dye content of all our diets would be 0.09 per cent, since our experience seemed to indicate a more rapid carcinogenesis at the higher level (e.g., compare diets 57 and 58 with 43 and 44).

Results.—The presence of thiouracil in diets 55 and 56, low in protein and riboflavin, resulted in an appreciable delay in tumor development (Chart 13). The last rat on diet 55 was killed after 500 days, and a sarcoma arising in the wall of a cysticercus cyst was found. The last rat on diet 56 was killed after 542 days still tumor-free. When the diets were repeated, there was no significant difference in rate of tumor development between rats on diets 44R and 56R up to 46 per cent tumor incidence, but there was a retardation of 75–100 days in tumor development with diet 55R (Chart 14).

Cirrhosis of some degree was present in the livers of all rats on diets 44 and 44R, whereas with the thiouracil-containing diets a few livers were not cirrhotic, and the severity of cirrhosis in the others was somewhat less than that in the controls. Moreover, cirrhosis was a little less severe in the rats that received thyroid than in those that got thiouracil without thyroid.

TABLE 6

COMPOSITION OF DIETS 50, 51, 52, 53, 54, 55, 56, 51R, 52R, 53R, 55R, 56R, 79, 80, 81, AND 82

	Diet 50	Diet 54	Diet 51	Diet 52	Diet 53	Diet 55	Diet 56
Casein, vitamin-free, Labco	100.0	200.0	200.0	200.0	200.0	100.0	100.0
Corn starch	770.0	669.0	674.0	674.0	679.0	775.0	775.0
Cottonseed oil	80.0	80.0	80.0	80.0	80.0	80.0	80.0
Items 4–12 in Table 6 as in Table 1 for all diets							
Riboflavin	0.002	0.008	0.008	0.008	0.008	0.002	0.002
Thiosalicylic acid	10.0	10.0					
Thiouracil			5.0	5.0		5.0	5.0
Desiccated thyroid				0.4			0.4
4-Dimethylaminoazobenzene	0.6	0.6	0.6	0.6	0.6	0.6	0.6

The amount of desiccated thyroid shown above is the amount that was determined finally to be best.

Repeated diets, marked with R, i.e., 51R, 52R, 53R, 55R, 56R, differed from the original diets in containing 0.09 per cent of the azo dye. Diets 79, 80, 81, and 82 corresponded to 44, 50, 53, and 54, respectively, but contained 0.09 per cent of the azo dye.

The 20 per cent casein diets contained 1 gm. of choline chloride and 0.01 gm of niacin/kg.

The experiment was repeated 3 years later with diets 79, 80, 81, and 82, identical with diets 44, 50, 53, and 54, respectively. Chart 12 shows that the results with diets 79 and 80 were similar to those with diets 44 and 50, tumor development with diet 80 falling steadily 35–40 days behind that with diet 79. The results with diets 81 and 82 were entirely unlike those obtained earlier. Eleven of 21 rats that got the thiosalicylic acid-containing diet developed liver tumors, and ten of these had done so by the 300th day. Only five of the controls (diet 81) developed tumors, but there was a high mortality rate from infections between the 200th and 240th days, and the first tumors developed earlier than the first ones with diet 82.

Thiouracil.—Thiouracil was added at a level of 0.5 per cent to the same two diets that were used with thiosalicylic acid, and, in order to be sure that any effects observed with diets 51 and 55 were not due solely to the antithyroid action of thiouracil, two additional diets (52 and 56) were used in which the antithyroid effect was nullified by desiccated thyroid. At first the thyroid content was 0.05 per cent, but this was excessive, and, after 140 days, it was reduced to 0.033 per cent. The new level was inadequate, and after 40 days it was increased to 0.04 per cent, where it remained, for this amount kept the rats in a state of apparent slight hyperthyroidism. Subsequent examination of their thyroid glands disclosed no evidence of the effect of thiouracil.

The experiment was repeated (diets 44R, 51R, 52R, 53R, 55R, and 56R), and after 4 months the amount of DAB was

The results obtained with the diets that contained more riboflavin and more casein (51, 52, 53, 51R, 52R, and 53R) were equivocal and need not be discussed. A peculiar circumstance occurred with diet 53R. Three rats were found to have sarcomas in the wall of cysticercus cysts of the liver after 407, 525, and 534 days, and the only other cysticercus sarcoma observed in our hundreds of DAB-treated rats developed in a rat on diet 55. None of these rats had as many as six cysticerci.

Hard, brown, rough, rounded or fusiform calculi were found in the bladder, ureters, or kidney pelves of nearly one-fifth of the rats that received thiouracil for 150 or more days. Some reached a diameter of 2.5 mm. and a length of as much as 1 cm. In a few instances the calculi interfered with the emptying of the bladder. Analysis revealed that nearly all calculi consisted entirely or nearly entirely of thiouracil.

György and Goldblatt (5) observed that thiouracil at the level of 0.1 per cent in the diet prevented development of dietary cirrhosis of the liver in rats and later were able to show that, for several antithyroid drugs, the efficiency in the prevention of cirrhosis paralleled goitrogenic potency (6). The beneficial effect was thought to be due to decreased metabolism. It is not clear just how far this explanation applies to our experiments. The rats on diets 51 and 55 obviously had hypothyroidism, and their thyroids were subsequently found to be greatly enlarged. Their food intake was less than that of the others, and it is possible that their intake of the carcinogen was inadequate. However, the rats on the thyroid-containing diets

consumed more food than did the controls, and yet in the original experiment had an appreciably slower tumor development than did the controls. It is not apparent why the result was less pronounced when the experiment was repeated.

DISCUSSION

The usual procedure of killing simultaneously all rats in an experiment at the expiration of a predetermined interval of time has two advantages over the one we used: (*a*) the experiments are never greatly prolonged and (*b*) the tumor incidence at the chosen time interval is ascertained with absolute accuracy. Our method, however, permits determination of tumor incidence repeatedly at intervals extending over weeks or months and gives a better idea of the degree of protection offered by a given diet. We thought this method constituted a more stringent test of the anticarcinogenic effect of the substances used, but this may not be true, and no comparison of the two methods has been made.

It will be noted that the carbohydrate used in the diets was starch. Our first diets contained dextrose, but in February, 1943, we had to replace it with starch. It seemed better not to introduce an additional variable in these experiments by changing back to dextrose when it again became available.

A record of the riboflavin content of the livers of our animals would facilitate interpretation of our results, and knowledge of the amount of intestinal synthesis of riboflavin permitted by the various diets would be useful. In view of the observations of Miller and associates (16) that the riboflavin level in the liver of animals given DAB-containing diets which permitted rapid tumor development had low average hepatic riboflavin levels, and that rats given DAB-containing but protective diets had appreciably higher hepatic riboflavin levels, it must be assumed that in our experiments where protection was observed it was due to high riboflavin content of the livers and that where no protection was observed the riboflavin content of the livers was low. The mechanism by which riboflavin exerts its protective effect has been studied recently by Mueller and Miller (21). Although their results leave no doubt that riboflavin constitutes part of the enzyme system that splits DAB to noncarcinogenic products, it is possible that riboflavin is concerned also in other phases of metabolism of the dye.

TABLE 7

LIVER TUMOR INCIDENCE AND MINIMUM LATENT PERIOD WITH VARIOUS DIETS

Diet	Date begun	No. of rats used	Effective total	Rats with tumors	Minimum latent period (days)
43	7- 6-45	25	21	21	160
44	7- 6-45	25	17	15	152
45	8-23-45	24	21	19	153
46	8-23-45	24	19	10	167
47	9-25-45	24	20	19	106
48	9-25-45	24	21	18	127
50	12- 4-45	24	8	7	202
51	12-14-45	24	20	5	180
52	12-14-45	30	20	13	196
53	12-14-45	24	17	10	256
54	2-18-46	24	5	1	422
55	2-18-46	34	10	9	200
56	2-18-46	42	16	12	130
57	7-19-46	24	22	21	89
58	7-19-46	24	22	21	109
51R	11- 6-46	24	14	8	274
52R	11- 6-46	24	16	5	247
53R	11- 6-46	24	16	6	274
44R	11-18-46	24	21	19	121
55R	11-18-46	24	14	8	220
56R	11-18-46	24	15	11	128
59	3-31-47	24	17	14	135
60	4- 1-47	24	17	16	125
61	4- 1-47	24	16	13	129
62	4- 4-47	24	19	9	147
63	4-10-47	24	20	18	133
64	4-11-47	24	18	17	124
65	4-22-47	24	20	16	121
67	4-25-47	24	19	15	175
68	4-25-47	24	19	13	259
69	4-29-47	24	22	21	171
73	10-16-47	24	22	19	119
74	10-16-47	24	21	16	133
75	10-20-47	24	13	10	157
59R	10-29-47	24	10	9	126
64R	11- 5-47	24	21	17	112
D 57*	3-16-48	30	28	28	113
E 57*	3-16-48	19	16	12	113
W 57*	3-16-48	24	22	20	128
C 57*	5- 4-48	26	13	11	141
H 57*	7-12-48	24	21	21	123
63R	7- 8-48	24	10	6	117
76	8- 9-48	24	19	18	109
77	8- 9-48	24	24	22	106
78	8- 9-48	24	17	11	148
79	12-28-48	24	20	19	102
80	12-28-48	24	18	15	118
81	12-28-48	24	21	5	123
82	12-28-48	24	21	11	171

* The letter preceding 57 refers to the strain of rat. See section "The Strain of Rat" of the paper.

SUMMARY

1. Folic acid at the levels of 0.02 mg and 1.9 mg/kg of diet had no effect on tumor formation or cirrhosis of the liver induced by DAB.

2. No unequivocal evidence was obtained that strain differences in rats influence susceptibility to DAB.

3. Tumors developed at the same rate with diets containing 5, 10, and 20 per cent of cottonseed oil.

4. Tumors developed more slowly with a diet containing 5 per cent of corn oil than with diets containing 10 and 20 per cent of corn oil.

5. Tumors developed more slowly with a diet containing 20 per cent of olive oil than with diets containing 5 and 10 per cent of olive oil.

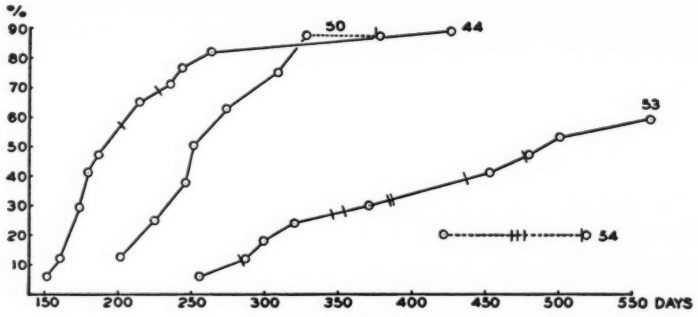


CHART 11.—Thiosalicylic acid. Diet 44=control, low riboflavin; diet 50=low riboflavin+thiosalicylic acid; diet 53=control, high riboflavin; diet 54=high riboflavin+thiosalicylic acid.

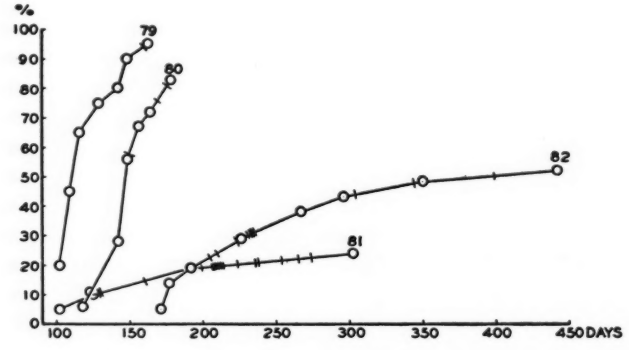


CHART 12.—Repeats of diets in Chart 11. Diet 79=old 44; diet 80=old 50; diet 81=old 53; diet 82=old 54

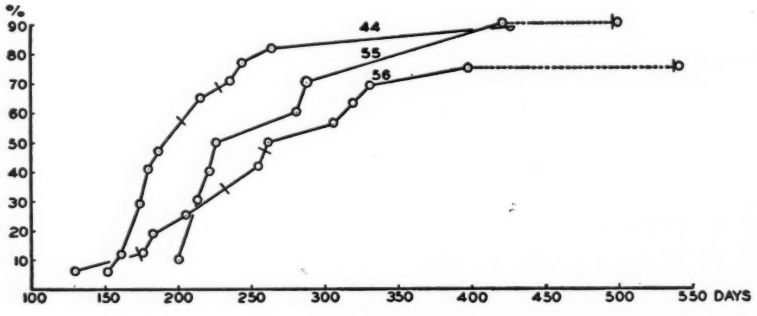


CHART 13.—Thiouracil. Diet 44=control, low riboflavin; diet 55=diet 44+thiouracil; diet 56=diet 55+desiccated thyroid

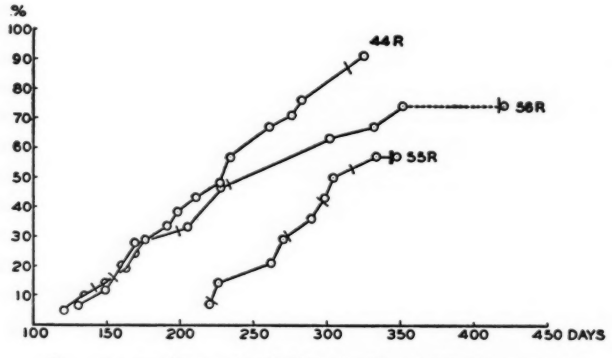


CHART 14.—Repeats of diets in Chart 13. *R*=repeat

6. On comparing results with the same concentrations of oils, tumor development was found to be slowest with diets containing 5 per cent of corn oil and 20 per cent of olive oil, but was essentially the same for the other concentrations and kinds of oil.

7. There was no difference in tumor development with diets low in riboflavin and containing 10 per cent and 30 per cent of casein, but it was greatly retarded with a 10 per cent casein diet high in riboflavin.

8. It appears that liver contains one or more factors other than riboflavin, pyridoxine, and biotin that influenced tumor development.

9. Thiosalicylic acid at the level of 1 per cent in the diet gave protection against tumor development but had no effect upon cirrhosis.

10. Thiouracil at the level of 0.05 per cent in the diet gave protection against tumor development and against cirrhosis of the liver. It is thought that this result was not due entirely to lowered intake of the carcinogen.

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The Estimation of Nucleic Acids in Individual Isolated Nuclei of Ascites Tumors by Ultraviolet Microspectrophotometry and Its Comparison with the Chemical Analysis

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Previous biochemical work by one (12, 13) of us has shown that the average amount of desoxyribonucleic acid (DNA) per cell, as compared to that of normal tissues, varies in different tumors. In certain tumors it was falling within the range which different authors have established to be characteristic for normal mouse tissues with a diploid cell population, while in other tumors the average DNA value per cell was significantly higher than this value. This elevation was not correlated with the mitotic index, but showed a certain rough parallelism with average nuclear size. In this and in earlier work by other authors (2, 5, 6, 15, 21), the importance of expressing amounts of nucleic acids (NA) in terms of average amounts per cell, instead of with dry or wet weights as basis, has been emphasized. The analysis of the ascites tumors has been performed by carrying out determinations on suspensions containing known numbers of cells or of nuclei and by computing the nucleic acid content for a single cell or nucleus. The ascites tumors presented certain advantages with regard to the difficulties in isolating nuclei from solid tumors and with regard to the absence of stroma and large amounts of necrotic tissue (6–8, 12, 13).

The computed amounts of nucleic acids per cell in the different ascites tumors represent naturally only an average value and do not give any information about the variability from cell to cell in the tumor cell population. In the cases where DNA was found to be elevated, the high average value might result from a mixture of different cell types with varying DNA contents, or the value might be characteristic for most cells of the particular tumor. An elucidation of this point can obviously come only from cytochemical technics which allow the analysis of *individual* cells for their nucleic acid content. Such a method is the ultraviolet microspectrophotometry as described by Caspersson in 1936 (4). With this technic, which utilizes the natural absorption of ultraviolet light at 257 mμ by the nucleic acids of the cells, it is possible to analyze in microscopical sections single cells for their nucleic acids. Recently, it was found by C. Leuchtenberger *et al.* (15) that isolated nuclei are a much more suitable material than tissue sections for the ultraviolet microspectrophotometry of nucleic acids, especially if a quantitative estimation of the DNA in individual cells is attempted.

Among other advantages which isolated nuclei have over whole cells in tissue sections and which have been discussed in detail in the previous paper (15), are the more homogeneous distribution of the absorbing material and the marked decrease in light scattering, both being of importance for microspectrophotometry. A comparison between the data on the DNA content of isolated nuclei of normal tissues by the ultraviolet microspectrophotometry and by the biochemical analysis on the same material showed a close agreement (15). Furthermore, the ultraviolet microspectrophotometry on individual cells revealed variations in the amount of DNA in the nuclei of the same tissue, as, for instance, in the rat liver, while the biochemical analysis on a mass of nuclei gave of necessity only an average value, which may be misleading, since it need not represent the content of any one individual nucleus (15).

It seemed of interest, therefore, to analyze isolated nuclei of tumors for their nucleic acids by ultraviolet microspectrophotometry and to compare the results with the biochemical data on the same material.

MATERIALS AND METHODS

For these studies the Ehrlich ascites tumor and the DBA ascites lymphoma were chosen.

Detailed data on these ascites tumors are to be found in previous publications (9, 11–13). For the present experiments, 20×10^6 Ehrlich ascites tumor cells were inoculated intraperi-

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toneally into hybrid albino male mice weighing 20–25 gm. The resulting ascitic fluids were collected 5–8 days after inoculation. About 60×10^6 lymphoma cells were inoculated into male DBA mice intraperitoneally, the resulting ascitic fluids being collected 8–12 days after inoculation. The cellular composition of the ascitic fluids was studied on fresh Papanicolaou smears; only those showing the typical picture of the well developed ascites tumor were used for the isolation of nuclei and subsequent chemical determinations of nucleic acids and proteins. For the studies of normal tissue, liver and sperm of freshly killed beef were used. Isolation of nuclei was carried out by the citric acid technic used by Vendrely and Vendrely (24). The final suspension of the nuclei was made with M/18 citric acid. Chemical determinations on suspensions containing known numbers of isolated nuclei were carried out as previously described (9). Nitrogen was determined by a micro-Kjeldahl technic, and the amount of protein calculated according to the formula used by Davidson and Leslie (5).

approximately diploid one of the DBA lymphoma is in good agreement with the earlier chemical determinations (12, 13).

A comparison between the ribonucleic acid content in the nuclei of the tumor and that of the normal cells shows that both tumors contain considerably higher amounts of nuclear RNA than do the normal cells (20–25 per cent, as compared to 3–7 per cent of the total nuclear NA). The relatively high amount of RNA in the tumor nuclei is all the more striking, since during the isolation procedure some of the RNA is lost from the nuclei (24).

Since the cytochemical data presented in Table 1 are *computed mean* values and, therefore, do not reveal the variations occurring from nucleus to

TABLE 1

AMOUNTS OF NUCLEIC ACIDS AND PROTEINS IN ISOLATED NUCLEI OF TUMORS AND NORMAL TISSUES BY CYTOCHEMICAL AND CHEMICAL ANALYSES

	Cytochemical analysis: Ultraviolet microspectrophotometry				Chemical analysis					
Material	No. of nuclei measured	NA* per nucleus in 10^{-9} mg.	DNA† per nucleus in 10^{-9} mg.	RNA‡ per nucleus in 10^{-9} mg.	NA* per nucleus in 10^{-9} mg.	DNA† per nucleus in 10^{-9} mg.	RNA‡ per nucleus in 10^{-9} mg.	Protein per nucleus in 10^{-9} mg.	$\frac{\text{RNA‡}}{\text{DNA†}}$	$\frac{\text{Protein}}{\text{DNA†}}$
Ehrlich ascites tumor	39	18.0±1.7	14.0±1.5	4.0±0.4	17.3	12.9	4.4	48.0	0.34	3.72
DBA lymphoma ascites tumor	30	8.8±1.9	6.8±0.6	2.0±0.2	8.2	6.6	1.6	27.9	0.24	4.23
Beef liver	42	5.9±0.11	5.9±0.11	—0—	6.7	6.2	0.5	21.5	0.08	3.46
Bull sperm	56	2.8±0.03	2.8±0.03	—0—	3.4	3.3	0.1	14.0	0.03	4.24

* NA = Total nucleic acid. † DNA = Desoxyribonucleic acid. ‡ RNA = Ribonucleic acid.

The microspectrophotometric studies were carried out on nuclei of the same suspension of isolated nuclei used for chemical analysis. The isolated nuclei were immersed for 1 hour in glycerol, put on quartz slides, and analyzed for nucleic acids by the ultraviolet microspectrophotometric photographic method as described previously (15). For the differentiation of DNA and RNA the nuclei were treated with Worthington crystalline ribonuclease (0.2 mg/1 cc distilled water) for 2 hours at 37° C. In order to free the enzyme from possible proteolytic activity, the ribonuclease was purified with saturated ammonium sulphate according to the procedure of M. McDonald (personal communication). From the difference in absorption between control nuclei (kept for 2 hours at 37° C. in distilled water) and ribonuclease-treated ones, the amounts of DNA and RNA were computed.

RESULTS

The results of the cytochemical and chemical analyses are presented in Table 1. The data show the rather close agreement between the values obtained by chemical analyses and the mean values of DNA and RNA obtained by ultraviolet microspectrophotometry.

It can be seen, furthermore, that, while the Ehrlich ascites tumor cell has twice the amount of DNA of that of the beef liver cell, the DNA content of the DBA lymphoma is approximately the same as that of the normal cell. This tetraploid DNA content of the Ehrlich ascites tumor and the

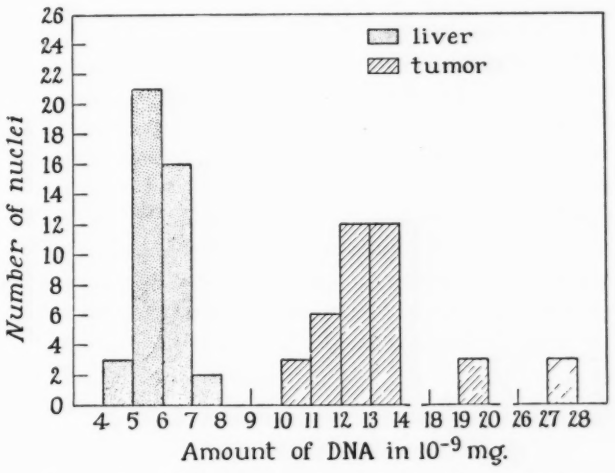

CHART 1.—DNA determined by ultraviolet microspectrophotometry in beef liver and Ehrlich ascites tumor nuclei.

nucleus, it seemed of interest to show the individual data on the DNA content of nuclei in beef liver and ascites tumor in the following histogram (Chart 1). From the histogram it can be seen that the great majority of the DNA values in beef liver nuclei (37 out of 42 measured) have a DNA content varying between 5 and 7×10^{-9} mg/nucleus; or, in other words, among this group the lowest

DNA value is approximately 30 per cent less than the highest, while the remaining nuclei outside of this range vary up to 50 per cent.

A similar variation in the DNA content can be seen to occur in the nuclei of the ascites tumor. While the amount of DNA per nucleus is approximately twice that of the beef liver, 35 out of 39 tumor nuclei measured show a DNA content of from 10 to 14 $\times$ 10^{-9} mg.—therefore, also, an approximate variation of 30 per cent.

DISCUSSION

The close agreement between the results of the microspectrophotometric and biochemical analyses on nucleic acid content of isolated tumor nuclei is in accordance with the earlier work on isolated nuclei of normal tissue (15). In both studies it was observed that, while the mean value of DNA per cell is approximately the same regardless of whether it is computed from the microspectrophotometric analysis of *individual* nuclei or from the biochemical analysis of a *mass* of nuclei, the microspectrophotometry reveals a variation in the content of DNA from nucleus to nucleus in normal as well as in tumor cells. Whether such differences in amounts of DNA from cell to cell within the same tissue are due to errors inherent in microspectrophotometry or are true biological variations cannot be decided with certainty at this time. However, the possibility exists, as discussed in detail in previous publications (16, 17), that these variations are, at least in part, biological ones. While such a suggestion is contrary to the opinion of some investigators (23, 18) who claim that the DNA content is the same for all cells of all somatic tissues of an organism and therefore independent of any metabolic function of the cells, recent findings by Leuchtenberger and Schrader (16) support the view that biological variations in DNA content might occur within a tissue. These workers observed that the nuclei of the salivary gland from the snail *Helix aspera* Müller carried widely varying amounts of DNA in cells of the same gland (in an order of magnitude of 1:30) and that the amount of DNA in a nucleus was closely related to the production of secretory granules in the cytoplasm. Though the differences of DNA as found in the present study are of course much less marked than those in the salivary gland nuclei, they might nevertheless be indicative of changes in metabolic activities or of polyteny of cells within the same tissue.

Ever since Boveri (3) suggested that tumors are composed of cells with a "faulty chromosome complement," it has been generally assumed that all tumors show variations in their nucleic acid content. The results presented in this study are only partially in accordance with such a view, namely, in regard to the RNA, but not if one considers the DNA. While the Ehrlich ascites tumor contains twice the amount of DNA of that of a normal cell, the DBA lymphoma shows a normal DNA value. Furthermore, cells with twice the DNA content occur also in normal tissues, as shown by previous work on rat liver, and are thus not a characteristic deviation of tumor cells. The conclusion from these data that the DBA lymphoma is a tumor with predominantly diploid nuclei, while the Ehrlich ascites tumor carries predominantly tetraploid nuclei, is supported by the findings of Hauschka and Levan (10), who have carried out metaphase chromosome counts on the same tumors. These workers observed that the chromosome numbers occurring with maximum frequency in the Ehrlich ascites tumor were around tetraploid, namely between 75 and 84, while the chromosome number of the DBA lymphoma gave a unimodal peak at 40, which is the diploid number of the mouse. Earlier investigations on the DNA content of mouse tumors (14) and studies on human tumors, now in progress, are in accordance with the concept that tumors are not characterized by special deviations in their amounts of DNA as compared to normal cells.

The picture is different, however, if we consider the RNA in the two ascites tumors. Both tumors show (in spite of the normal DNA content of the lymphoma) a marked increase of RNA in their nuclei, as compared to the RNA of nuclei of normal cells as seen in Table 1, where the RNA/DNA ratios are listed. Arneson and co-workers (1) found a similar augmentation in the RNA content in leukemic as compared to normal spleen nuclei, though the DNA content of the leukemic spleen nuclei was normal. Petermann and Schneider (19) also observed a pronounced increase of RNA in the nuclei of a transplanted mouse leukemia over that of normal spleen nuclei; in this case the DNA content of the leukemic cells was 45 per cent higher than the DNA of the normal cells.

On the basis of these various results it seems that the RNA content of the tumor nuclei so far examined is higher than that of normal nuclei, regardless whether the DNA is increased or not. Whether all tumors show such a high value of RNA in their nuclei and whether this is characteristic for tumors only or holds also for other rapidly growing tissues must await further studies. But the apparently consistent higher values of RNA in the tumor nuclei thus far examined are rather remarkable if one considers the probability that a part of the RNA is lost from the nuclei during the isolation procedure.

On the basis of the relatively high RNA/DNA

ratios in tumor nuclei, one might expect a similar increase in the protein/DNA ratio, but, as can be seen from the last column of Table 1, the protein/DNA ratio in tumor nuclei is rather close to the protein/DNA ratio of normal nuclei. These findings are in accordance with the results of other workers (19). Since it has been demonstrated that considerable amounts of proteins are extracted from the nuclei during the isolation procedures (21), the rather constant protein/DNA ratios in isolated nuclei of various tissues is of interest. One might speculate that the protein left in the nuclei after isolation is very stable because of its linkage with the DNA which is not affected at all by the isolation procedure. Perhaps we deal here with a protein which might be a type of "chromosomal protein." Studies on Arvelius by Schrader and Leuchtenberger (22) clearly indicate two different types of proteins in nuclei: the so-called "chromosomal proteins" which are linked to the DNA and the "extrachromosomal proteins," the synthesis of which seems independent of the DNA and which is possibly concerned with the metabolic processes of the cells.

SUMMARY

1. The results of the microspectrophotometric analysis on nucleic acids in isolated nuclei of tumors and normal tissues were in good agreement with those of the biochemical analysis on the same material.

2. The DNA content/cell in the Ehrlich ascites tumor is approximately *twice* that found in normal diploid nuclei (corresponding to tetraploid nuclei), while the relative *deviations from the mean value* do not differ significantly from that found in normal cells. The DBA ascites lymphoma contains amounts of DNA similar to those of normal diploid cells. These results are in agreement with the chromosome counts of Hauschka and Levan on the same material.

3. The average RNA content per nucleus was markedly increased (20–25 per cent of the total nucleic acid) in the ascites tumors over that of the nuclei of normal tissue (3–7 per cent of the total nucleic acid).

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The Viability and the Average Desoxypentosenucleic Acid Content of Micronuclei-containing Cells Produced by Colchicine Treatment in the Ehrlich Ascites Tumor*

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The appearance, after colchicine treatment, of cells containing large numbers of micronuclei—also called chromosome-vesicles, idiomeres, karyomeres—has been observed by several authors in different biological materials. Brues and Jackson (2) found them in regenerating rat liver and interpreted their formation to be due to the swelling of the individual chromosomes after mitosis has been arrested by the drug. All intermediate stages between the increase in the size of the chromosomes and the fully developed micronuclei were found. With the use of animal cells, similar findings have been reported by Ries (18) in axolotl epidermis, by Tennant and Liebow (24) in tissue cultures of mammary cancer, by Schairer (19) and confirmatively by Dittmar and Maas (4) in the Ehrlich ascites tumor. Lettré (14) failed to produce large numbers of micronuclei-containing cells after colchicine treatment in the Ehrlich ascites tumor, but obtained them with great regularity and in high frequency after injections of N-methyl-colchicamide. In this experiment he observed that, while arrested metaphases reached a maximal frequency 16–20 hours after treatment, multinucleate cells started to appear later and were at maximum 24–25 hours after the injection of the drug. He, as all other authors quoted, interpreted the formation of micronuclei similarly to Brues and Jackson as a reconstruction of resting nuclei from individual dispersed chromosomes or small groups of chromosomes.

In plant cells, micronuclei-containing cells and the eventually reconstructed resting nuclei have been shown to become polyploid (16). For animal cells, two different opinions were expressed as to the probable number of chromosome sets contained by the micronuclei-filled cells. Ries, in axolotl epidermis, found that the micronuclei gradually fuse into a single resting nucleus. This resting nucleus was of the same size as nuclei in the original material containing the diploid number of chromosomes. Tennant and Liebow, on the other hand, working with tissue cultures of mammary cancer, state that one cell filled with micronuclei probably contains a chromosome moiety that falls to the lot of two daughter cells normally. The latter authors also found that their mammary cancer cultures remained viable and transplantable to animals in spite of the profound nuclear changes that have taken place after colchicine treatment.

The present investigation was concerned with the changes in the viability and chromosome ploidy of tumor cells after the appearance of large numbers of micronuclei. The Ehrlich ascites tumor was selected as test material because of the established relationships between the number of viable tumor cells inoculated and the survival time of the hosts (5, 8, 11) and because its chromosome ploidy (7) and average desoxypentosenucleic acid content per cell (5) have been determined previously.

PROCEDURE AND RESULTS

Treatment.—In all experiments, unless otherwise stated, groups of ten hybrid male albino mice weighing 20–25 gm. received inoculations of 0.1 cc. undiluted ascitic fluid of the Ehrlich ascites tumor, containing about 13×10^6 cells. In each experiment two groups were treated by intraperitoneal injections of colchicine (dissolved in 0.9 per cent NaCl), while one was kept as control. One of the treated groups served for observing survival time, while the other was sacrificed around median survival time and used for differential counting of cell types, determination of average nucleic acid content per cell, and further inoculation of known numbers of tumor cells. All these procedures were carried out by the same methods as were used previously (5, 10). Treatment with colchicine was started on the third day after inoculation and was continued with daily doses of 12 μg/20 gm mouse until the seventh day, when the dose was increased to 16 μg. and continued until the death of the animals. The controls were injected with corresponding volumes (0.1–0.2 ml.) of saline.

Morphological changes.—While on the average 0.9 per cent of all tumor cells in the untreated Ehrlich ascites tumor are bi- or multinucleate (10), ascitic fluids collected at median survival time from fourteen animals treated with colchicine as described above contained an average of 45.4 per cent multinucleated tumor cells (standard error 5.3 per cent, range 19.3–82.7 per cent; see also Table 2). The frequency of mitotic figures was only

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slightly elevated. The multinucleated cells often contained a great number of micronuclei, varying in number and size, frequently showing a vesicular appearance and enclosing a nucleolus (Figs. 1–7). An increased inflammatory reaction was present, the average frequency of nontumorous cells being 43.8 per cent of all cells, while the corresponding figure was 22.5 per cent in fourteen saline-treated controls. Polymorphonuclear leukocytes were mainly responsible for this increase.

Survival time of treated animals.—It was found previously (5, 8, 11) that the inoculation of different numbers of Ehrlich ascites tumor cells produces different survival curves—different not only quantitatively, but also with regard to their shape. Low numbers of cells did not lead to ascites development in 100 per cent of the animals but partly to solid tumor development with increased inflammatory reaction. Injection of high numbers of cells resulted in short survival times with little variability and ascites development in 100 per cent. It was considered necessary to investigate separately the effect of colchicine treatment on animal groups that were injected with high doses of cells and on those injected with low doses of cells. The doses selected to be inoculated were those which would produce 100 per cent mortality within a short period with ascites tumors (15×10^6 tumor cells) and those near the "critical" cell number, i.e., the lowest dose to produce typical ascites tumor in 100 per cent of the animals inoculated ($0.6–1.0 \times 10^6$ tumor cells). It can be expected, on basis of previous experiments (11), that an agent lethal to a certain percentage of tumor cells will cause great changes in median survival time and in the shape of the survival curve of animals injected with the critical number of cells; while the change, if any, will be smaller in animals injected with doses as high as 15×10^6 cells. Colchicine treatment and the injection of the control groups were carried out as described above.

TABLE 1

EFFECT OF COLCHICINE TREATMENT ON MEDIAN SURVIVAL TIME

Series	No. tumor cells inoculated	Approximate median survival time in days, control group	Approximate median survival time in days, colchicine-treated group	Prolongation of median survival time in per cent
A	15×10^6	7	13	86
B	1×10^6	11	16	45
C	1×10^6	11	18	64
D	0.6×10^6	20	29	45
E	0.6×10^6	22	33	50

As shown in Table 1, colchicine markedly prolonged the median survival times of the treated groups. This confirms the findings of Lettré (13). The prolongation was brought about in a similar way in both experimental groups, those with high and with low doses of tumor cells. Mortality curves of the treated and control groups had rather similar shapes in all cases after the point where the first animal died. Ascites developed in 100 per cent of all control and treated animals. A typical curve (group *C*) is shown in Chart 1.

Implications of these results will be discussed below.

Survival time of animals injected with micronuclei-containing cells.—Three ascitic fluids from colchicine-treated animals were selected containing 61.6, 62.0, and 74.9 per cent micronuclei-filled tumor cells, respectively. About 600,000 tumor cells were injected into mice of three groups, each containing ten animals. This dosage was selected because it was previously found (11) that in this range a decrease of 20 per cent in the number of inoculated viable cells results in pronounced changes of survival times and ascites development. Ten animals were each injected with 600,000 tumor cells from one of the control animals. The survival time of all four groups was observed. Median survival times were 18.5, 16, 15, and 17.5 days for the three treated groups and the control group, respectively. Neither these median survival times nor the shape of the survival curves revealed any significant difference between the groups. Thus, the fact that most of the tumor cells were filled with micronuclei did not impair to any major degree their proliferative capacity after inoculation.

Colchicine treatment through several transplant generations.—In order to test whether, as found with some other growth-inhibiting agents (12), a resistance could be developed towards the effect of colchicine in the tumor line used, the following experiments were carried out:

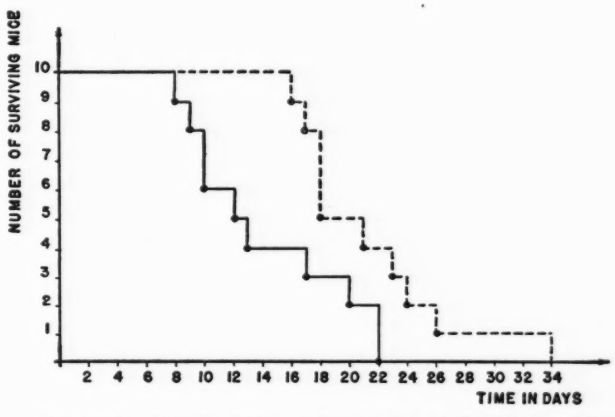

CHART 1.—Survival times of mice injected with 1 million Ehrlich ascites tumor cells. ●—●—●: control, treated with saline injections; o---o---o---o: group treated with colchicine as described in text.

Serial transplantations of the Ehrlich ascites tumor were performed through twelve successive transfer generations. To each generation 2–4 million tumor cells were inoculated intraperitoneally into groups of ten mice. The animals were treated with colchicine by daily injections of 12 μg/20 gm mouse during all twelve transfer generations. As soon as any of the animals showed marked swelling of the abdomen, it was killed, the ascitic fluid collected, the total cell number per milliliter determined, and the cell types present differentially

counted. Inoculations of known numbers of cells were then carried out.

The appearance of micronuclei was detected regularly on all transfer generations. While there were fluctuations in their relative frequency, these fluctuations did not seem to be correlated to the serial number of the transfer generation and were perhaps dependent on variations in the dosage of the inoculum and time of ascites collection.

At the end of the twelfth transfer generation, 3.1×10^6 tumor cells were inoculated into twenty mice. Ten of the mice were treated with colchicine, as in the previous transfer generations, while ten received injections of saline. Simultaneously, twenty mice were inoculated with 3.25×10^6 tumor cells from the untreated control line of the Ehrlich ascites tumor. Ten of these were treated with colchicine, as described above, while the remaining ten were injected with saline.

In this experiment a definite prolongation of survival times could be observed in both colchicine-treated groups, as compared to their controls. This prolongation was very similar in the tumor line previously treated with colchicine through twelve transfers and in the untreated tumor line. In the former, median survival time was prolonged from 9 to 16 days and in the latter from 11 to 18 days. Thus, no evidence could be obtained for a decreased sensitivity of the tumor to the effect of colchicine after it had been treated with the drug during twelve transfer generations.

Desoxypentosenucleic acid (DNA) content of ascitic fluids with high percentages of tumor cells containing micronuclei.—Fourteen ascitic fluids were collected at median survival time from different colchicine-treated groups, containing high percentages of micronuclei-filled tumor cells. Of the control groups, fourteen ascitic fluids were selected at random. In all cases, the total number of cells was counted in a Buerker hemocytometer, cell types were differentially counted on Papanicolaou smears, and pentosenucleic and desoxypentosenucleic acid (PNA and DNA) were determined according to the procedure of Schmidt and Thannhauser (20). DNA was not calculated as a difference between total nucleic acid and PNA, but was determined separately (21). The results are shown in Table 2. As the content of inflammatory cells was variable and often high, a correction had to be made with regard to their previously established (5) lower DNA content. This correction was performed by using the previously obtained average DNA value of 0.7×10^{-6} μg. DNAP per inflammatory cell (Table 2, col. 5). The corrected values were then compared with:

TABLE 2

DESOXYPENTOSENUCLEIC ACID (DNA) CONTENT OF COLCHICINE-TREATED EHRLICH ASCITES TUMORS

	No. of samples	Per cent inflammatory cells	Multinucleated cells per 100 tumor cells	Av. DNA per cell 10^{-6} μg. P	Same as col. 4, after correction for inflammatory cells*	Calculated average DNA per cell on basis of hypothesis I†	Calculated average DNA per cell on basis of hypothesis II†
	1	2	3	4	5	6	7
Control	14	22.5±3.0	0.9‡	1.29±0.06	1.48±0.08	1.41‡	1.40
Colchicine-treated	14	43.8±4.2	45.4±5.4	1.21±0.04	1.62±0.10	2.04±0.08	1.40

t test for treated and control of col. 5: $t = 1.12$, $P > 0.2$.
t test for treated samples of cols. 5 and 6: $t = 3.54$, $P < 0.01$.
t test for treated samples of col. 6 and control of col. 5: $t = 5.11$, $P < 0.001$.
} P: probability that the difference is due to chance.

* Correction: $\frac{\text{DNA per cell} - 0.7 \times \text{per cent inflammatory cells}}{\text{Per cent tumor cells}}$

† Hypothesis I: Multinucleate tumor cells contain an average of 2.8×10^{-6} μg. DNA P.
Mononucleate tumor cells contain an average of 1.4×10^{-6} μg. DNA P.
Hypothesis II: All tumor cells contain an average of 1.4×10^{-6} μg DNA P.

‡ Mean values of previous determinations (10).

a) similarly corrected values of fourteen control determinations (col. 5).

b) hypothetic values, calculated on the assumption that micronuclei-containing cells contain double amounts of DNA (col. 6). It was previously found that the average value of DNA per cell in the untreated Ehrlich ascites tumor is about 1.4×10^{-6} μg. DNAP, a value suggesting the presence of tetraploid population. That the Ehrlich ascites tumor really represents a mainly tetraploid cell population has since been confirmed by Hauschka and Levan by metaphase chromosome counts (7). It was assumed, for each treated fluid, that mononucleate tumor cells contain an average of 1.4 and micronuclei-filled cells 2.8×10^{-6} μg. DNAP. The mean value per cell, on the basis of this hypothesis, was calculated for each case and compared to the values actually found.

The following conclusions can be drawn from Table 2:

a) No significant difference can be found between the control and treated group of col. 5; i.e., the average DNA content per cell of the treated

and control tumors after correction has been made for inflammatory cells ($P > 0.2$).

b) The difference between the values for the colchicine-treated groups of cols. 5 and 6 is significant ($P < 0.01$), indicating that the hypothesis on basis of which col. 6 was calculated and which implies that micronuclei-filled cells contain twice as much DNA as mononucleate tumor cells does not fit the values experimentally found.

c) A comparison between the control group of col. 5 and the treated group of col. 6 reveals a difference of high significance ($P < 0.001$). This means that, if the above hypothesis were true, a significant difference could be expected between the DNA content of the treated population and the controls. As stated above under (*a*), such a difference was not found.

These determinations make it seem highly probable that the micronuclei-containing cells have average amounts of DNA that are similar to those of the untreated, mainly mononuclear tumor cells and definitely not twice as high.

The PNA determinations were not useful for calculations as were made for DNA, because the PNA content of the particular inflammatory cell populations in treated and control groups is unknown and cannot be assumed with any degree of probability to be identical with the values found in experimental peritonitis.

DISCUSSION

In agreement with the previous findings of Lettré (13) and of Brodersen (1), the survival time of mice which received inoculations of different numbers of Ehrlich ascites tumor cells was found to be prolonged after treatment with repeated small doses of colchicine. To be effective, treatment had to be started soon (by the third day) after inoculation. This prolongation of life was, however, of a different character than that which was previously found after treatment with some other drugs—e.g., trypaflavine (9). After trypaflavine treatment, the survival curve of animal groups inoculated with a certain number of cells resembled that of animals inoculated with smaller numbers and untreated. This means that, if the "critical" cell number was chosen which was the lowest necessary to produce ascites development within an uniformly short time in all animals, trypaflavine treatment prolonged survival times, made them considerably more variable, and led in many cases to development only of solid tumors, without ascites formation. These same results occur without treatment if the number of cells inoculated were less than the "critical." This may be tentatively explained by assuming that trypaflavine actually kills part of the proliferating tumor cells. Different conditions are met when analyzing the effect colchicine had on survival times. In the latter case, no "qualitative" change of the survival curves could be observed. Median survival time and the individual survival times were prolonged, but the shape of the survival curves was similar to that of the controls. Ascites developed in 100 per cent of the animals after treatment, even when the "critical" cell number was used for inoculation. The first death occurred later than in the control groups, but the subsequent course was unchanged. A possible explanation could be that colchicine had merely a delaying effect on cell division soon after inoculation, without any considerable part of the cells being killed. Arrest of metaphase, occurring mainly after the first few injections during the course of the prolonged treatment, may be the mechanism by which the growth is slowed down in the beginning. Later this gives way to the development of micronuclei that are generally believed to represent the transition stage between the arrested metaphase and the eventually reconstructed resting nucleus. In good agreement with the concepts above are the findings that the main morphological effect of prolonged colchicine-treatment in the Ehrlich ascites tumor is the production of micronuclei-filled cells, which, upon inoculation into new untreated hosts, show the same proliferative capacity as untreated cells of the control line. Trypaflavine treatment, on the other hand, resulted in severe morphological damage (9), and the injection of treated cells to untreated animals led to longer survival times with more solid tumors and less ascites development than the injection of the same cell number from the control line.[1]

Law and Boyle (12) recently reported the development of resistance toward certain folic acid analogs in different lines of transplanted mouse leukemia after treatment with the substances in question through four to five transfer generations. Our experiments, aiming at the development of a colchicine-resistant subline of the Ehrlich ascites tumor in an analogous way, gave negative results. After continued treatment with colchicine during twelve transfer generations no change was observed in the sensitivity of the cells towards the drug, as judged by micronucleus production and prolongation of survival times.

As mentioned in the introduction of this paper, different opinions were expressed as to the chromosome ploidy of the micronuclei-containing cells compared to corresponding untreated material. In

[1] G. Klein, unpublished data.

recent years, evidence has been obtained for the existence of a correlation between the average desoxypentosenucleic acid content per cell of certain cell populations and the distribution of chromosome ploidy in such populations (3, 6, 15, 23). On the basis of present knowledge, it could be reasonably expected that a doubling of the chromosome set in a cell will be reflected by an increase of the desoxypentosenucleic acid content to approximately twice the original value. No evidence for the existence of such an increase could be obtained in our colchicine-treated tumor cell populations that contained high frequencies of micronuclei-filled cells. This indicates that, in the material used, the chromosome ploidy of such cells is unchanged and similar to that of the untreated population. Furthermore, since such cells are generally believed to develop directly from arrested metaphase figures, their failure to contain double amounts of DNA has to be explained by one of the following two alternatives: either the cells of the Ehrlich ascites tumor do *not* double their DNA content until early metaphase under control conditions or this doubling would occur normally but is inhibited by colchicine. The second assumption is perhaps made less probable by the findings of Skipper *et al.* (22), who showed that colchicine is relatively ineffective as an inhibitor of nucleic acid synthesis. The doubling of DNA has been recently claimed to occur after telophase in some species (17, 23). Such a mechanism might be a possible explanation of the present results.

SUMMARY

1. In agreement with previous reports of other authors, colchicine was found to prolong the survival times of mice injected with different cell doses of the Ehrlich ascites tumor if treatment was carried out with repeated small doses and started soon after inoculation. The effect is probably due to an initial inhibition of tumor cell division and not to the death of any appreciable numbers of tumor cells.

2. High frequencies of micronucleus- (karyomere-, idiomere-, chromosome vesicle-) containing tumor cells were present in the ascitic fluids of treated animals around median survival time. The viability and proliferative capacity upon further inoculation of these cells was not decreased to any detectable degree.

3. Prolonged treatment through twelve subsequent generations with colchicine produced no decrease of sensitivity to colchicine of the tumor used.

4. Average desoxypentosenucleic acid (DNA) content of ascitic fluids containing high frequencies of multi-micronuclear cells did not differ significantly from the controls, indicating that no increase in chromosome ploidy has occurred in these cells that are believed to develop directly from arrested metaphases. The implications are discussed.

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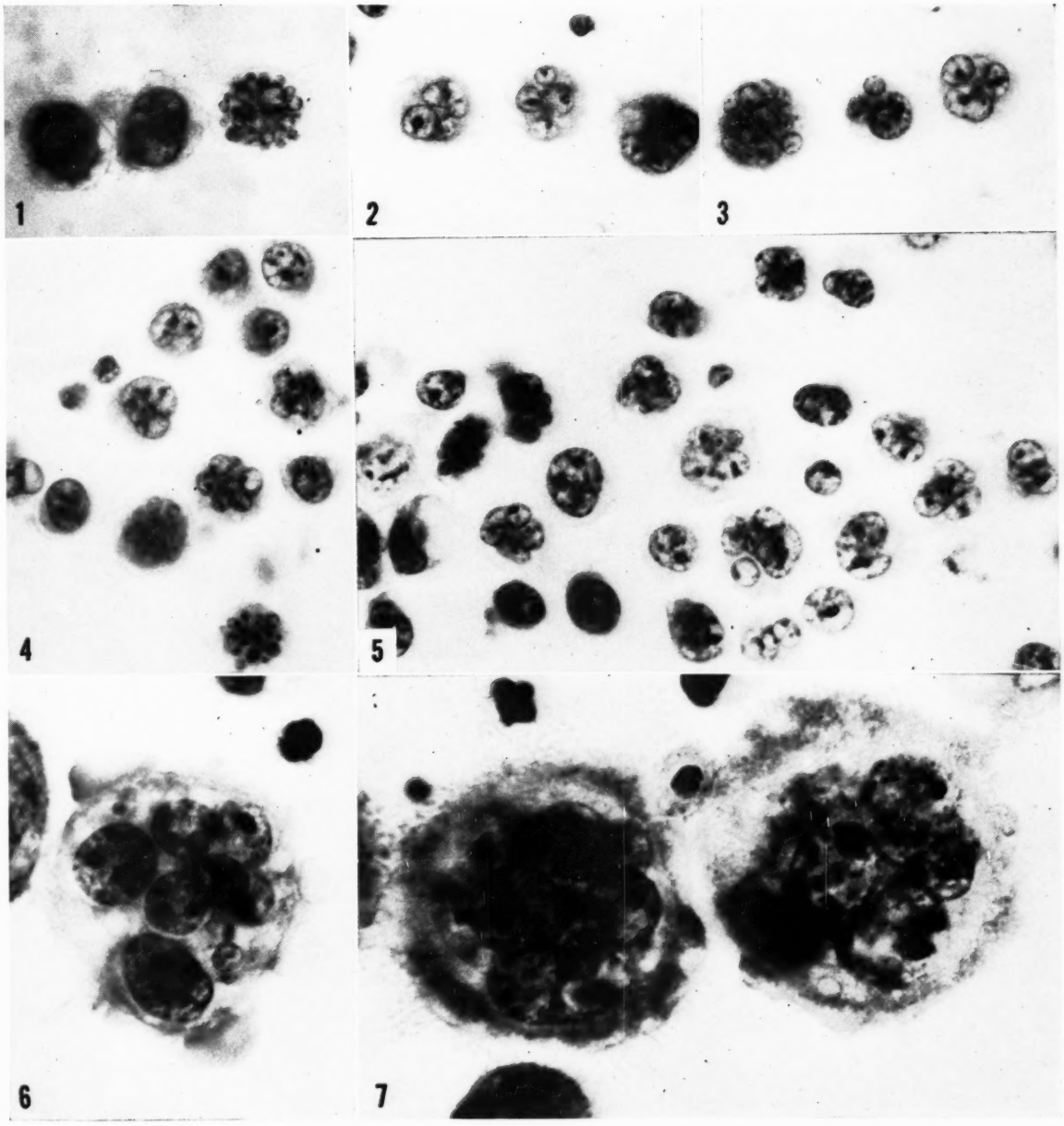

FIGS. 1–7.—Papanicolaou's smears of Ehrlich ascites tumor after prolonged treatment with colchicine. Note cells containing several micronuclei. Magnification: Figs. 1–5, ×700; Figs. 6–7, ×1400.

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The Significance of the Anterior Chamber in Tumor Transplantation. III. Natural Resistance and Acquired Immunity*

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The literature contains conflicting data on the extent to which strain barriers to transplanted tumors can be overcome by the use of the anterior chamber of the eye as a transplantation site. Lushbaugh and Steiner (5) observed the same incidence of progressive growth of a maligant lymphoma following subcutaneous and anterior chamber inoculation. Eichwald *et al.* (2) observed progressive growth of mouse neuroblastoma C1300 in 16 per cent of C57 brown mice following subcutaneous transplantation, while after anterior chamber inoculation the incidence rose to 61 per cent. Greene (4) has stated that strain barriers "appear to be largely negated when the anterior chamber is used" as the transplantation site.

A transplanted tumor, similar to a skin graft, must overcome two types of resistance: (*a*) a "natural" resistance which may prevent the establishment or "take" of the tumor. The nature of this type of resistance is complex and not entirely understood. It is influenced by species and strain differences, preceding immune processes, local immune phenomena, the stromal and vascular response to the graft, and others; (*b*) an acquired immunity, representing the response of the host to the tumor, and which may cause its regression after it has grown for a certain period of time. If strain barriers were abolished by use of the anterior chamber, both the natural and the acquired resistance would have been overcome. The tumor could then take in the anterior chamber of a foreign host with as much ease as in a random site in mice of the strain of origin; it would grow progressively in a similar number of animals, in most instances about 100 per cent; it would grow at a similar rate, and the survival time would not differ appreciably; finally, the metastatic spread would be similar to that in the strain of origin.

The data presented in this paper were obtained by transplanting several mouse tumors into the anterior chamber and the subcutaneous tissue of various mouse strains. They indicate that, while the natural resistance is often by-passed by use of the anterior chamber, the acquired immunity is not overcome. The data also demonstrate that transplantation to the anterior chamber is a useful tool for differentiating natural resistance from acquired immunity to the growth of transplanted tumors.

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MATERIALS AND METHODS

Tumors.—

1. Mouse neuroblastoma C1300, originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and carried in this laboratory for over 30 generations by subcutaneous transplantation to strains A and ABC mice.

2. Mouse mammary carcinoma C3HBA, obtained through the courtesy of Dr. A. Towbin, Columbus, Ohio, and carried in C3H mice.

3. Spontaneous mammary carcinoma of strain CBA mice.

4. Spontaneous AK lymphoma; both (3) and (4) were obtained through the courtesy of Dr. T. F. Dougherty, Salt Lake City, Utah.

Mouse strains.—

1. Strain A mice, obtained from Bar Harbor, Maine, or bred in this laboratory by brother-sister matings, and their backcrosses with F_1 hybrids (ABC).

2. C57 brown mice, subline CD, obtained from Bar Harbor, Maine.

3. C57 black mice, obtained from Bar Harbor, or bred in this laboratory by brother-sister matings.

4. CFW mice, obtained from Carworth Farms, Inc., New City, N.Y.

5. CBA mice bred by brother-sister matings for over 30 generations, and obtained through the courtesty of Dr. T. F. Dougherty, Salt Lake City, Utah.

Method of transplantation.—As described previously (2).

Pattern of experiments.—Unless stated otherwise, similar numbers of mice were inoculated in the anterior chamber and in the subcutaneous tissue. Some of these were sacrificed between the sixth and the fifteenth day for histological determination of tumor growth. The remaining mice—referred to below as "survivors"—were observed for the development of progressively growing tumors. Differences were considered significant when the P value was 0.05 or less.

Tumor measurements.—The tumor size was determined by multiplying the largest diameter by the largest diameter at right angles to the former, disregarding depth. The mean value of the largest measurements of the positive animals is reported below as "Mean Maximum Size."

EXPERIMENTS

Transplantation of neuroblastoma C1300 to strain of origin (A, ABC).—Following subcutaneous inoculation the tumor grew progressively in 89.4 per cent of the mice; following anterior chamber inoculation it grew in all mice (Table 1). The difference is significant (P = 0.01). Regression of tumors was not observed in either group. The incidence of tumor growth did not differ significantly between A and ABC mice. The mean survival time and mean maximum size of the positive animals of both groups are shown in Table 2. Many subcutaneously inoculated mice and almost all mice of the anterior chamber group developed large metastases in the regional lymph nodes. Visceral metastases were not seen.

Transplantation of neuroblastoma C1300 to C57 brown mice.—Growing tumor was found in only 22 per cent of the sacrificed mice of the subcutaneous group but in almost all mice of the anterior chamber group (Table 1). In the survivors of the subcutaneous group the incidence of tumor growth did not differ significantly from that in the sacrificed mice. In contrast, in the anterior chamber group there was a significant drop in the incidence of tumor growth from sacrificed animals to survivors (P = 0.01). It indicates that regression of tumors established in the anterior chamber was fairly common. It usually occurred after the tumor had perforated the eye. Progressive tumor growth still was significantly more frequent than in the subcutaneous group (P < 0.001).

The survival periods of the positive mice of both the subcutaneous and anterior chamber groups exceeded those of the corresponding ABC mice (P < 0.01, < 0.01) (Table 2). In both groups the tumors also reached a larger size than in the corresponding ABC mice. C57 brown mice did not develop metastases.

Transplantation of neuroblastoma C1300 to C57 black mice.—Growing tumor was found in only 15 per cent of the sacrificed mice of the subcutaneous group; in the anterior chamber group it was present in the great majority of mice (Table 1). Regression of subcutaneously established tumors was uncommon, as shown by the incidence of progressive tumor growth in the survivors. In contrast, regression of tumors which had taken in the anterior chamber was very common, as shown by the very significant difference in the incidence of tumor growth between the sacrificed mice and the survivors (P < 0.001). Regression usually occurred before the tumors had perforated the eye. Progressive tumor growth in the survivors was not signifi-

TABLE 1

TRANSPLANTATION OF NEUROBLASTOMA C1300 TO VARIOUS MOUSE STRAINS

	Subcutaneous inoculation*				Anterior chamber inoculation*			
	Progressive	Growth on sacrifice (day)			Progressive	Growth on Sacrifice (day)		
Mouse strain	tumor growth	6–9	10–15	Total	tumor growth	6–9	10–15	Total
ABC (A)	194/217 (89.4)				56/56 (100)			
C57 brown	52/249 (20.9)	6/28 (21)	6/26 (23)	12/54 (22)	171/238 (71.8)	17/20 (85)	28/29 (97)	45/49 (92)
CFW	16/32 (50)				22/33 (67)			
C57 black	11/107 (10.3)	5/23 (22)	1/18 (6)	6/41 (15)	17/99 (17.2)	15/21 (71)	10/11 (91)	25/32 (78)

* Numbers in parentheses indicate percentages.

TABLE 2

SURVIVAL, TUMOR SIZE, AND METASTASES FOLLOWING TRANSPLANTATION OF MOUSE NEUROBLASTOMA C1300 TO VARIOUS MOUSE STRAINS*

	Mean survival (days) of positive mice		Mean maximum size (mm.²)		Lymph node metastases after
	Subcutaneous	Anterior chamber	Subcutaneous	Anterior chamber	Anterior chamber
Mouse strain	S.E.	S.E.	S.E.	S.E.	inoculation†
ABC (A)	39.0±1.1	39.4±0.8	543± 36.7	228±19.5	53/56 (95)
C57 brown	57.7±2.3	50.5±1.2	807± 47.2	391±13.3	0/238 (0)
CFW	55.2±6.3	46.9±2.4	980±123.6	378±45.9	0/33 (0)
C57 black	65.0±5.3	49.4±4.1	708±110.3	208±34.9	0/99 (0)

* See "Materials and Methods."

† Numbers in parentheses indicate percentages.

cantly more common than in the subcutaneous group.

The survival periods of the positive mice of both the subcutaneous and anterior chamber groups significantly exceeded that of the corresponding ABC mice ($P = 0.01$, $= 0.05$). Metastases were not seen (Table 2).

Transplantation of neuroblastoma C1300 to CFW mice.—The incidence of progressive tumor growth did not differ significantly in the subcutaneous and anterior chamber groups but was significantly below that of the corresponding ABC groups ($P < 0.001$, < 0.001) (Table 1). There were no sacrifice groups. The survival periods of the positive mice of the subcutaneous and anterior chamber groups significantly exceeded that of the corresponding ABC groups ($P < 0.05$, $= 0.05$) (Table 2). Metastases were not seen.

Transplantation of Ak lymphoma to CBA mice.—The donor tumor was in its first transplant generation. Growing tumor was found more commonly in the anterior chamber than in the subcutaneous group, but all takes regressed (Table 3). Prior to regression in the survivors, several of the anterior chamber tumors invaded beyond the eye (microscopically proved).

Transplantation of mammary cancer C3HBA to ABC mice.—Growing tumor was found more commonly in the anterior chamber than in the subcutaneous group, but all tumors regressed (Table 3). Prior to regression about half of the survivors of the subcutaneous group developed tumors exceeding 0.5 cm. in diameter, while almost all survivors of the anterior chamber group developed tumors invading beyond the eye.

Transplantation of spontaneous CBA mammary cancer to CBA mice.—The donor tumor was far advanced, exceeding 3 cm. in its largest diameter. The recipients were of the same strain, had an equal sex distribution, were about 1 year old, and free of spontaneous tumors at the onset of the experiment. Growing tumor was found in none of the sacrificed mice of the subcutaneous group. (Four mice with autologous subcutaneous transplants of similar tumors showed growing tumor at the transplant site at the time of death from their spontaneous tumors.) Growing tumor was found in all sacrificed mice of the anterior chamber group with homologously transplanted tumors (Table 3). Eleven of 28 survivors of the subcutaneous group developed progressively growing tumors, some of which may have been spontaneous. Only three of 27 survivors of the anterior chamber group developed progressively growing tumors. In one of the three mice two periods of temporary regression were observed. In several of the other mice the tumors invaded extensively beyond the eye before they regressed. The sex of the hosts did not influence the extent of tumor growth.

TABLE 3

TRANSPLANTATION OF TUMORS OTHER THAN NEUROBLASTOMA C1300

		Subcutaneous inoculation				Anterior chamber inoculation			
		Progressive	Growth on sacrifice (day)			Progressive	Growth on sacrifice (day)		
Tumor	Strain	tumor growth	6–9	10–15	Total	tumor growth	6–9	10–15	Total
Ak Lymphoma	CBA	0/36	1/4	0/4	1/8	0/36	3/4	1/4	4/8
Mammary cancer C3HBA	ABC	0/27	1/4	0/4	1/8	0/29	3/4	1/4	4/8
Spont. CBA mammary cancer	CBA	11/28	0/2	0/2	0/4	3/27	2/2	2/2	4/4

DISCUSSION

The "negation" of strain barriers.—Use of the anterior chamber as a transplantation site lowered the strain barrier to transplanted tumors. The tumors took in the anterior chamber of most mice of the strains studied, while in the subcutaneous tissue this was observed in a significantly lower number of mice. This also applies to a tumor (C1300) transplanted to mice of the strain of origin (A, ABC).

Use of the anterior chamber *lowered* strain barriers to transplanted tumors but *failed to abolish* them. Tumors established in the anterior chamber regressed in: a minority of mice (C1300 to C57 brown); in about half (C1300 to CFW); in the majority (C1300 to C57 black); or in all mice (Ak lymphoma to CBA, C3HBA to ABC). They failed uniformly to metastasize, in contrast to anterior chamber transplantation of C1300 to A (ABC) mice. The survival periods of the positive animals in the foreign strains following anterior chamber transplantation exceeded those of the strain of origin.

Natural resistance and acquired immunity.—Regression of tumors established in the anterior chamber could be due either to the development of an immune state or to invasion of tissue beyond the anterior chamber where further growth does

not occur as readily as in the "sanctuary" of the anterior chamber. The latter interpretation is unlikely, since tumors in the anterior chamber regress without apparent relation to the extent of invasion of the structures beyond the anterior chamber. This applies to tumors transplanted to the anterior chamber of heterologous hosts; these almost always regress before the anterior chamber is filled with tumor tissue. It also applies to homologous hosts in which tumors often regress after invasion of the periocular tissues has proceeded for a considerable period. On the other hand, most investigators agree that the anterior chamber participates in immune events (1, 3, 6, 7), at least in an attenuated degree. The most attractive explanation of the regression of tumors established in the anterior chamber, then, is the development of an immune state. While use of the anterior chamber can by-pass the natural resistance characteristic of certain sites (e.g., the subcutaneous tissue), it cannot eliminate the effects of acquired immunity.

The fate of a tumor transplanted to the anterior chamber is therefore determined essentially by the strength of the immune response alone, since the natural resistance in this site is sufficiently weak to permit establishment of the tumor in the large majority of mice. The proportion of mice without tumors among the survivors of an anterior chamber group to those with progressively growing tumors is an index of the strength of the immune response. In contrast, the fate of a tumor transplanted to the subcutaneous tissue is determined by the combined effects of natural resistance and acquired immunity. Failure to grow progressively is due either to nonestablishment or to the development of a sufficiently strong immune state. Progressive growth can occur only when both natural resistance and acquired immunity are weak. Therefore, subcutaneous transplantation is not a suitable means of determining the strength of the immune response.

In the experiments reported, the natural resistance and the acquired immunity varied in strength with different strains and also with mice of the same strain. Both the natural resistance and the acquired immunity of CBA mice to Ak lymphoma were strong: subcutaneously inoculated tumors took in only few instances, and all tumors (subcutaneous and anterior chamber) regressed. The natural resistance of ABC mice to mammary cancer C3HBA was relatively weak, and many subcutaneously inoculated tumors took; the immune response, however, was strong, and all tumors regressed. The natural resistance of C57 black mice to neuroblastoma C1300 was strong, and only few tumors took after subcutaneous inoculation. There also was a strong immune response, and the tumors regressed in the great majority of mice with anterior chamber transplants. The natural resistance of C57 brown mice to neuroblastoma C1300 was also strong, and only a minority of the subcutaneously inoculated tumors took; the immune response, however, was relatively weak, and the majority of tumors inoculated to the anterior chamber grew progressively. The natural resistance of A (ABC) mice to neuroblastoma C1300 was very weak, and almost all subcutaneously inoculated tumors took; there was no evidence of an immune response, and all tumors inoculated to the anterior chamber grew progressively. Surprisingly, regression of CBA mammary cancer was observed in several CBA mice with anterior chamber transplants; it would seem to indicate that mice of this strain can acquire immunity to a tumor from a mouse of a closely related genetic background.

The incidence of progressive tumor growth will differ between a subcutaneously inoculated group of mice and another with anterior chamber transplants only when the natural resistance in the subcutaneous tissue is stronger than the immune response (transplantation of neuroblastoma C1300 to C57 brown and to ABC mice). When the natural resistance and the acquired immunity are equally strong, the incidence will not differ (neuroblastoma C1300 to C57 black mice). When the natural resistance is weaker than the immune response, the incidence of progressive tumor growth will not differ in the two groups of mice (mammary cancer C3HBA to ABC mice); the tumors will take subcutaneously in many mice, but regression will occur according to the strength of the immune response, which also determines the incidence of progressive tumor growth in the anterior chamber group.

Correlation between natural resistance and acquired immunity.—There often was a positive correlation between the natural resistance in the subcutaneous tissue and the acquired immunity of a strain to a given tumor. Both the natural resistance and the acquired immunity to neuroblastoma C1300 were the strongest in C57 black mice; they were weaker in C57 brown mice, and the weakest in A (ABC) mice. There is evidence that a positive correlation between natural resistance and acquired immunity also existed in mice of the same strain. It was most apparent in C57 black mice with transplants of neuroblastoma C1300: the tumors took in 78 per cent of mice of the anterior chamber group, but regressed in the great majority. Following subcutaneous inoculation the tumors took in only 15 per cent of the mice, but very few of these regressed. It suggests that the few mice of

this strain with a sufficiently weak immune response to permit progressive growth also had a sufficiently weak natural resistance in the subcutaneous tissue to permit the tumor to take in this site. A similar situation probably pertains to C57 brown and CFW mice with transplants of neuroblastoma C1300.

SUMMARY

1. To determine the extent to which strain barriers are lowered by use of the anterior chamber as a transplantation site, several mouse tumors (neuroblastoma C1300, Ak lymphoma, C3HBA mammary carcinoma, CBA mammary carcinoma) were transplanted to various mouse strains (A, ABC, C57 brown, C57 black, CFW, CBA).

2. The barriers of all strains were lowered: the tumors took in the anterior chamber of the majority of mice of all foreign strains; this did not take place after subcutaneous transplantation.

3. The extent to which strain barriers were lowered differed with the tumor and the strain. In no instance were strain barriers abolished. When the tumors grew progressively the survival periods were prolonged, and metastatic dissemination did not occur. However, the tumors often regressed, presumably due to the development of a resistant state.

4. It is surmised that the natural resistance of a foreign host to a tumor can be largely by-passed by use of the anterior chamber but that the barrier of acquired immunity is not overcome.

5. Transplantation to the anterior chamber is a convenient means of testing the strength of the immune response of a host to a tumor. Subcutaneous transplantation is less suitable for this purpose, since many tumors fail to take and therefore cannot demonstrate the development of a resistant state.

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Uptake of Peptides by a Free-Cell Neoplasm*

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The present communication deals with the question whether peptides can enter living cells without hydrolysis. When partially hydrolyzed proteins are injected intravenously, large portions of the peptides they contain are excreted in the urine. The extent of this loss is almost independent of the injection rate (3, 4, 12), indicating a rather sharp division of peptides into those quickly utilized and those not utilized, with little evidence for slowly utilized peptides. Possibly only those peptides for which there is strong extracellular peptidase activity are retained. Peptides are not important transport forms of amino acids from the intestinal mucosa to other tissues during the digestion of protein (2, 8).

Seven peptides were studied: glycylglycine, glycylglycylglycine, L-leucylglycine,[1] L-phenylalanylglycine,[1] L-tyrosylglycine,[1] glutathione, and α-L-glutamyl-L-glutamic acid.[2] The first of these is known to be utilized upon injection (7); the last was found in the present study to be largely metabolized by the rat. The cells studied included the Ehrlich mouse ascites carcinoma cells and human and duck erythrocytes. The red cells failed to take up any of the peptides. Most of the peptides were hydrolyzed by suspensions of the neoplastic cells; with leucylglycine, phenylalanylglycine, and tyrosylglycine the cleavage was too fast to permit observation of cellular uptake of unsplit peptide. Glutathione was recovered quantitatively (as bound glycine) in the suspending fluid, being rapidly oxidized to the disulfide form. Therefore, these data are limited to glycylglycine, glycylglycylglycine, and glutamylglutamic acid.

METHODS

The propagation, incubation, separation, and analysis of the cells have been described (5, 6, 11). The cellular glutathione was removed with lead acetate (10) before analysis for glycine and with basic lead carbonate as an overlayer in the alumina absorption column (1) before analysis for glutamate. It was shown that large quantities of glutathione and glutamine did not interfere with the determination of either free or bound glutamate under the conditions of analysis. α-Glutamylglutamic acid was found to be quantitatively absorbed on the alumina column and eluted, along with free glutamic acid, and the dipeptide converted quantitatively to glutamic acid by hydrolysis in 4 N hydrochloric acid for 16 hours at 110° C. The following experiment summarized these observations: A solution containing 0.068 μM glutamate, 0.037 μM glutamylglutamate, 0.274 μM glutamine, and 0.326 μM glutathione was passed through the alumina column and elution then carried out as usual after washing. The resulting solution was then hydrolyzed as described above. 0.149 Micromoles of glutamate were found, compared to the original 0.142 (0.068 as glutamic acid, 0.074 as glutamylglutamic acid). This dipeptide was also satisfactorily recovered after addition to rat urine and to trichloroacetic acid filtrates of human plasma. The same hydrolytic conditions were used to release bound glycine. Amino acid concentrations were calculated in mM/kg of water. The carcinoma cell was assumed to contain 80 per cent water on the basis of previous experiments.

TABLE 1

RETENTION OF α-GLUTAMYLGLUTAMATE IN THE RAT AFTER INJECTION

Intraperitoneal (Exp. 1) or intracardial (Exp. 2) injection of dipeptide in neutral solution into rats fasted 24 hours. Composition of the urine during the 12 hours previous to the injection compared to that in the 7–11 hours following the injection.

Exp.	Free glutamic acid excreted (μM/hr)	Bound glutamic acid excreted (μM/hr)	Extra bound glutamic acid (μM)	Recovery of bound glutamate injected (per cent)
1. Before injection	2.8	20.6		
After injection	2.6	25.1	52	8.5
2. Before injection	5.8	39.3		
After injection	10.9	49.7	78	14.5

RESULTS

Table 1 records the small losses of glutamylglutamate into the urine occurring after intraperitoneal and intracardial injection into the rat. Table 2 reports observations of experiments in which glutamylglutamate was incubated with the suspensions of ascitic carcinoma cells from the mouse (6.7–10 mM of dipeptide per liter of suspension). Control portions of the cells incubated in the ascitic plasma without dipeptide addition con-

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tained appreciable concentrations (2.1–2.9 mM) of bound glutamate, but clear-cut increases occurred upon addition of the dipeptide. Approximately half as high a level of extra bound glutamate was reached inside the cells as outside; hence, there was no evidence that the uptake was concentrative. During the 2-hour incubation period, 44–72 per cent of the added glutamylglutamate disappeared, most of it reappearing as free glutamate. An unexpected effect of the presence of the dipeptide was the nearly complete loss of the ability of the carcinoma cells to concentrate *free* glutamate, the distribution ratios (cellular)/(extracellular) falling from 10 or 15 to about unity.

Table 3 presents the results with glycylglycine and glycylglycylglycine. Large increases in bound glycine within the carcinoma cells were seen upon incubation with these peptides. Bound glycine disappeared during the experiment to the extent of 19–44 per cent, most of the loss being accounted for by formation of free glycine. It should be noted that glycylglycine may have been the peptide entering the cells when triglycine was added. The two glycine peptides inhibited the maintenance of the concentrative activity for free glycine. This effect was observed also with duck erythrocytes, the distribution ratios falling from 3.6 to 0.9 (with glycylglycine) and to 0.6 (with triglycine); the peptides were added at a 2 mM level. No entrance of these two glycine peptides into erythrocytes could be detected, nor was there clear-cut entrance of glutamylglutamate into the red blood cells.

DISCUSSION

The results indicate that three peptides can be taken up by the neoplastic cells in peptide form as well as after hydrolysis. There was evidence for extensive hydrolysis within the cells, since cells resuspended in Krebs' Ringer-bicarbonate medium also split the three peptides rapidly. Whether any direct anabolic utilization of the peptides occurs cannot be said. Greater uptake of peptides by the cells might have been observed if the portions taken up had not been partially metabolized. Failure to observe accumulation of a peptide by cells

TABLE 2

DISTRIBUTION OF FREE AND BOUND GLUTAMATE UPON INCUBATION OF CARCINOMA CELLS WITH α-GLUTAMYLGLUTAMIC ACID

6.7–10 mM dipeptide (plus $NaHCO_3$ to neutralize) added per liter ascitic fluid suspensions; incubation time, 2 hours. Concentrations are mM/kg of water

	Free glutamate			Bound glutamate		
Exp.	In medium	In cells	Distribution ratio	In medium	In cells	Distribution ratio
1:						
A. Incubated control	0.27	2.67	10.0	0.22	2.94	
B. Experimental sample	8.01	7.14	0.89	4.79	5.21	
Extra bound glutamate				4.6	2.3	0.5
2:						
A. Incubated control	0.33	4.92	15.0	0.20	2.11	
B. Experimental sample	7.58	8.79	1.2	15.0	11.1	
Extra bound glutamate				14.8	9.0	0.61

TABLE 3

DISTRIBUTION OF FREE AND BOUND GLYCINE UPON INCUBATION OF CELLS WITH GLYCYLGLYCINE AND GLYCYLGLYCYLGLYCINE

6–9 mM dipeptide added per liter ascitic fluid suspension; incubation, 1 hr. in exp. 4; 2 hr. in exp. 5. Concentrations are mM/kg water

	Free glycine			Bound glycine		
Exp.	In medium	In cells	Distribution ratio	In medium	In cells	Distribution ratio
3:						
A. Incubated control	0.29	5.7	19	0.10	0.67	
B. Sample with diglycine	1.45	10.3	7.1	21.8	5.5	
Extra bound glycine				21.7	4.7	0.22
4:						
A. Incubated control	0.28	5.9	21	0.08	0.11	
B. Sample with diglycine	0.63	11.1	18	10.5	3.9	
Extra bound glycine				10.4	3.8	0.36
C. Sample with triglycine	4.0	33.7	8.3	18.6	4.4	
Extra bound glycine				18.5	4.3	0.23

does not exclude its uptake, unless, as with oxidized glutathione, all the added peptide can be recovered in the extracellular phase.

The uptake of these peptides was strikingly inferior to that of free amino acids. Every α-amino acid tested, whether of natural occurrence or not, or of the L- or D-configuration, has been found to be concentrated by these neoplastic cells. The failure of the glycine peptides to be concentrated is of particular interest, because glycine itself is so strongly concentrated. The inhibitory effects of free amino acids upon the concentrative uptake of other amino acids is related to the extent to which the inhibitor is itself concentrated.[3] The three peptides are unusual in being strong inhibitors of amino acid uptake without being themselves concentrated. Gale and Van Halteren (9) have suggested that peptide formation may be involved in the accumulation of amino acids by *S. aureus*.

SUMMARY

α-L-glutamyl-L-glutamic acid was excreted to only a minor extent by way of the urine after injection into the rat. This dipeptide was taken up by the Ehrlich mouse ascites carcinoma cells upon incubation *in vitro*, as indicated by increases in bound glutamate, by a procedure having a degree of specificity for glutamylglutamic acid. Increases in bound glycine in the carcinoma cells, not attributable to glutathione or protein, occurred upon incubation with glycylglycine or triglycine, indicating that these two peptides were probably also taken up in peptide form. Each of the three peptides underwent considerable hydrolysis. All three peptides strongly inhibited the maintenance of the normal concentration gradient for the contained free amino acid.

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The Coenzyme I Oxidase System in Normal and Tumor Tissues*

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To date, no detailed comparative study of the series of respiratory enzymes which constitute a system in the oxidation of reduced diphosphopyridine nucleotide (coenzyme I or DPN) in normal and tumor tissues has been published. The importance of this system, however, has found expression in the literature, which contains ample evidence that a considerable portion of oxidative cellular metabolism is carried out through the nicotinamide nucleotide components.

Studies of certain parts of this oxidative system, with particular reference to the metabolism of tumors, have been limited. Euler and Hellström (7) found diaphorase and cytochrome oxidase present in Jensen sarcoma. Lockhart and Potter (21) observed that in three types of neoplasms there was no oxidation of $DPNH_2$ in the absence of added cytochrome c but found that the reaction took place in the presence of additional cytochrome c. While numerous investigators have studied the terminal portion of this respiratory system, no comprehensive investigation has correlated these data with the initial part of the system; and, consequently, conclusions concerning the relative abilities of various tissues, normal and neoplastic, to catalyze the over-all aerobic oxidation of $DPNH_2$ have not been established. This is due, in part, to the fact that the constitutive elements and mode of operation of certain parts of the chain have not been clarified definitively. Also, the study has been rendered difficult because of the failure to obtain some of the intermediates in soluble and stable form.

The system designated as coenzyme I oxidase can be resolved into the following known enzyme components: (*a*) diaphorase, a flavoprotein catalyzing the direct oxidation of $DPNH_2$, (*b*) cytochrome c reductase, which mediates the oxidation between $DPNH_2$ and cytochrome c, and (*c*) cytochrome oxidase, which facilitates the oxidation of reduced cytochrome c. There is no general agreement as to what entities constitute the second portion of this chain, but it has been established that factors operating in this unit link the enzymatic removal of hydrogen from $DPNH_2$ with the transfer of electrons to cytochrome c. Thus, in keeping with the general system as defined by Slater (35), cytochrome c reductase, as considered in this treatise, includes the activity of diaphorase, together with the essential elements which accomplish the oxidation of reduced diaphorase and the reduction of cytochrome c.

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MATERIALS AND METHODS

Enzyme preparation.—The tissue extracts were prepared by a modified method of Keilin and Hartree (16). This consisted essentially of the extraction of the tissue with phosphate buffer and the precipitation of the enzymes with acetate buffer. A major difference in the two preparations, however, was the ability of the extracts prepared by the modified method to reduce cytochrome c enzymatically. This was absolutely essential in a system intended to measure total coenzyme I oxidase activity. Retention of the cytochrome c reductase activity was probably achieved by eliminating the copious washings prescribed by the method of Keilin and Hartree. While the enzyme preparation was not completely free from hemoglobin, its presence did not interfere in the spectrophotometric studies, since the enzyme preparation was also introduced into the "blank" cell.

As already noted, the comparative study undertaken in the present investigation was aimed to analyze total coenzyme I oxidase activity of cells. Schneider and Hogeboom (28) observed that by far the majority of cytochrome oxidase activity in mouse liver was found in the mitochondrial cell fraction. These authors (12, 13) have also shown that the presence of $DPNH_2$-cytochrome c reductase is not restricted to any definite cell structure. The greatest amount of this enzyme is found in the mitochondria and submicroscopic particles but was also present in the nucleus. Since the individual enzyme components of the coenzyme I oxidase system were found in various sedimentable and nonsedimentable fractions, only a general tissue preparation was studied, and no attempts were made to analyze cell fractions obtained by the methods of differential centrifugation.

The method of preparing the enzyme was as follows: Mice maintained on a standard Rockland mouse pellet diet were killed by exsanguination. The tissues were removed immediately, weighed, and extracted with cold M/50 phosphate buffer, pH 7.2, 0.5 cc. being used for every 0.1 gm. of tissue. The extraction was accomplished by grinding the tissue in a mortar with a small amount of purified sand. Care was taken to rinse the mortar thoroughly with a small amount of the buffer, but the combined volumes used for extracting and rinsing did not

exceed the amount calculated above. The mixture was centrifuged in a refrigerated centrifuge at 2,000 r.p.m. for 5 minutes and the supernatant fluid collected. The residue was washed with one-half the volume of phosphate buffer used in the original extraction and the mixture again centrifuged. The supernatant fluid was combined with that of the first step. Ice-cold acetate buffer, pH 4.6, 4 times the volume of phosphate buffer used in extracting the tissue, was added to the combined supernatant fluids and the mixture allowed to stand for a few minutes in an ice bath to permit the coagulation of the precipitate. The mixture was again centrifuged in the cold, and the supernatant was discarded. The residue was then taken up in a volume of phosphate buffer, pH 8.5, equal to the original volume of phosphate buffer, pH 7.2, used in the first step of extraction. Precipitation with acetate buffer was repeated. To the residue obtained after centrifugation was added the same volume of phosphate buffer, pH 8.5, used in the preceding step. The tissue preparation was kept on ice until use (within 2 hours after preparation).

Nonenzymatic reduction of DPN.—The nonenzymatic method of reducing DPN was based on the procedure of Potter as described by LePage (20). The reduction, however, was accomplished anaerobically, and the solvent for the coenzyme was alkaline phosphate instead of sodium carbonate–sodium bicarbonate mixture. The following are details of the method:

Ten mg. DPN was dissolved in 1.6 cc. phosphate buffer, pH 8.5. The sodium dithionite solution was similarly prepared —10 mg/1.6 cc phosphate buffer, pH 8.5; 0.96 cc. of the DPN solution was placed in the hollow stopper of a Thunberg tube and the same volume of the sodium dithionite solution in the tube proper. The tube was evacuated for 5 minutes. The contents of the tube were then mixed and incubated at 38° C. for 15 minutes; 16.08 cc. of glass-distilled water was added and the mixture aerated for 15 minutes or oxygenated for 5 minutes, in order to oxidize any remaining dithionite. The oxidized preparation used in the "blank" absorption cells was prepared as above, except that the reducing agent was replaced by water. The product proved to be somewhat unstable but was found satisfactory for special parts of this study if used immediately after preparation.

Enzymatic reduction of DPN.—In reducing DPN enzymatically, glutamate activated by its specific dehydrogenase was the reducing system. The dehydrogenase was prepared from acetone powder of mouse liver as follows: Fresh mouse liver was weighed and extracted with cold distilled water (3 cc/gm tissue) by grinding with purified sand in a mortar. This mixture was poured into 3 volumes of cold acetone and the precipitate filtered on a Buchner funnel. It was then immediately rubbed up with a small volume of cold water (5 cc/gm tissue), transferred to a cellophane dialyzing bag, and dialyzed against distilled water at 0° C. for 12 hours. The supernatant fluid obtained by centrifuging the mixture at 2,000 r.p.m. for 10 minutes contained glutamic dehydrogenase.

The procedure used for reducing DPN was that of Slater (34), with the exception that 1.0 cc. of the glutamic dehydrogenase solution described above was used instead of the 0.4 cc. of liver extract of Slater's preparation. Accordingly, the volume of 0.05 M Na_2HPO_4 was reduced to give the final volume specified in Slater's procedure. The $DPNH_2$ so prepared was used within 2 hours of preparation.

Measurement of enzyme activities.—Spectrophotometric studies were carried out with a Beckman DU spectrophotometer. The enzyme preparation was diluted in glass-distilled water, 1 cc/30 cc. This dilution was made immediately prior to the experimental run, since the diluted enzyme preparation was rather unstable. Slater (34) reports a similar lack of stability in his heart-muscle coenzyme oxidase preparation. The spectrophotometric determinations were carried out at 24° C.

The experimental reaction mixture for measurements of the total coenzyme oxidase system was made up of the following: (*a*) 1.5 cc. diluted enzyme, (*b*) 1.0 cc. $DPNH_2$ solution prepared by nonenzymatic reduction or 0.2 cc. $DPNH_2$ solution prepared by reduction with the glutamate system, and (*c*) glass-distilled water to give a final volume of 3.0 cc. In systems containing nonenzymatically reduced substrate, the "blank" Beckman cell contained enzyme and oxidized DPN. In experiments involving the use of enzymatically reduced $DPNH_2$, the "blank" contained enzyme and all the constituents used in the preparation of the substrate, except $DPNH_2$. Among these was nicotinamide, which was added to suppress the activity of the pyrophosphatases described by Kornberg and Lindberg (17).

Diaphorase determinations which required the presence of KCN and methylene blue were made with the same concentration of enzyme as used in the total system; 0.3 cc. of 2×10^{-3} M KCN and 0.1 cc. 5.3×10^{-4} M methylene blue were added to both experimental and "blank" cells. The final volume of the reaction mixtures was 3.0 cc.

In the cytochrome c reductase studies, the concentration of KCN was as used in the diaphorase system. This, together with cytochrome c, was added to the "blank" also. Cytochrome c was either a commercial preparation or a product prepared by the method of Keilin and Hartree (15). The experimental results obtained for any given tissue preparation were independent of the cytochrome preparation. In all spectrophotometric determinations, $DPNH_2$ was the last constituent added to the absorption cells. Optical densities were read within 15 seconds of the mixing time.

RESULTS AND DISCUSSION

Coenzyme I oxidase activity.—Preliminary results obtained by a colorimetric procedure devised to test the activity of the system under investigation revealed quantitative differences in reaction between two transplantable sarcomas and one transplantable adenocarcinoma, as compared to five normal tissues of mice. The method of assay was based on the ability of $DPNH_2$ to decolorize 2,6-dichlorophenol indophenol, as reported by Haas (10). Though this reaction was nonspecific, the results were considered of sufficient significance to warrant a more detailed investigation of the reaction.

With the more definitive spectrophotometric method, the coenzyme I oxidase activity of the normal and tumor tissues was re-examined. The results are summarized in Chart 1, which represents the total system unfortified by additional amounts of cytochrome c. The rates of activity for the respective tissues were found to be in general agreement with the data obtained in the aerobic colorimetric analysis. It is noteworthy that the results obtained in the study of normal tissues were not peculiar to any one animal strain, inasmuch as comparable results were obtained in the study of C3H, Rockland, and A mice. The low oxidative response of the transplantable sarcomas and adenocarcinomas was also substantiated. These values, however, fell essentially within the range of normal muscle, an observation which is in keeping

with the rather general finding that many enzyme systems in tumor tissue approximate the lower values for normal tissues. Of greater significance was the observation that hepatoma 98/15 in C3H mice showed a greatly diminished oxidative capacity when compared to normal liver.

Before investigating the specific causes for the failure of the coenzyme I oxidase system in tumor tissues, it was necessary to establish the enzymatic nature of the oxidation in question, since Singer and Kearney (32) have reported the nonenzymatic reduction of cytochrome c by pyridine nucleotide, as catalyzed by various flavins. The

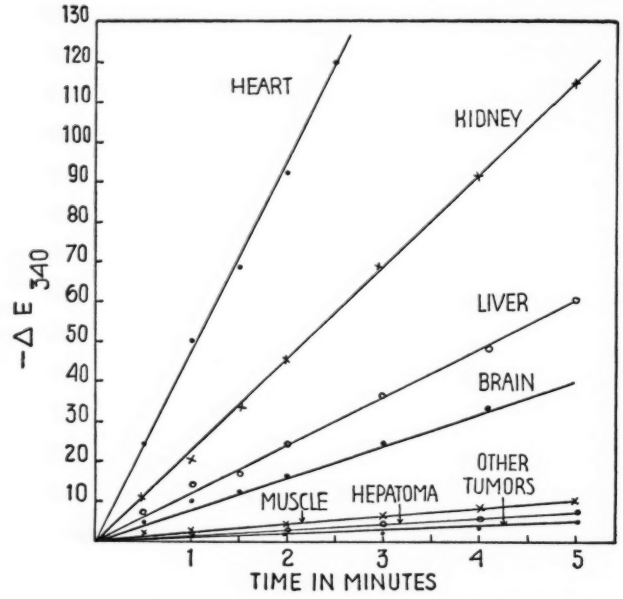

CHART 1.—The coenzyme I oxidase activity of normal and tumor tissues of mice. The tumors represented by a single line include Sarcoma 180, Sarcoma 37, dbrB adenocarcinoma, and an adenocarcinoma specific for C3H mice. $-\Delta E_{340}$ values represent the decrease in optical densities $\times 1{,}000$.

possibility of a nonenzymatic reduction was disproved, however, since heated enzyme preparations (90° C. for 5 minutes) of any one of the normal or tumor tissues failed to decrease the optical density at 340 mμ over a period of 15 minutes.

At this point, the possibility was entertained that the lowered oxidative response of tumors was peculiar to a system containing enzymatically reduced substrate. It was conceivable that similar studies, employing a substrate prepared by reduction with sodium dithionite, would yield quantitatively different responses. Pertinent to this idea was Slater's notation (34) that $DPNH_2$ prepared by a nonenzymatic modified method of Ohlmeyer (23) was unsatisfactory for certain parts of his oxidative studies. As illustrated in Table 1, the activity of certain tissues was not dependent on the type of substrate preparation. This was true of the less active tissues, viz., tumors, muscle, and brain. With the more active heart, liver, and kidney, oxidative values were considerably lower when the nonenzymatically reduced substrate was used. Presumably this was due to the interference of the products of oxidation of the reducing agent. The undesirability of the nonenzymatically reduced substrate was experimentally demonstrated in systems containing methylene blue and, more especially, in systems containing both methylene blue and cyanide. In such instances the inherent rate of autoxidation of the substrate was accelerated. This effect was also noted by Slater (34), who stated that the reason for this reaction is not known. Thus, though the nonenzymatically prepared substrate would have simplified the system under consideration, its disadvantages were such as to require the use of enzymatically reduced $DPNH_2$ in most experiments. Unless otherwise indicated, therefore, the data presented in this report were obtained with the latter kind of substrate.

TABLE 1

COMPARISON OF THE COENZYME I OXIDASE ACTIVITY OF NORMAL AND TUMOR TISSUE WITH ENZYMATICALLY AND NONENZYMATICALLY REDUCED $DPNH_2$.—ΔE_{340} IN 5′
(Decrease in optical densities × 1,000)

Tissue	Enzymatically reduced $DPNH_2$	Non-enzymatically reduced $DPNH_2$
Heart	230	155
Kidney	112	80
Liver	60	33
Brain	40	34
Muscle	8	8
Adenocarcinoma in C3H mice	6	6
dbrB adenocarcinoma in DBA mice	6	6
S-180	8	6
S-37	9	6
Hepatoma	7	8

Diaphorase and cytochrome oxidase studies.—In investigating the coenzyme I oxidase system for the factors essentially associated with the failure of the system in tumors, quantitative determinations of the diaphorase activity of the various normal and tumor tissues were first undertaken. Experimental systems designed to measure this activity contained cyanide to inhibit the cytochrome oxidase portion of the respiratory chain and methylene blue to by-pass the cytochrome system. It has been established that methylene blue is capable of oxidizing reduced diaphorase. Cyanide was employed at a final concentration of 2×10^{-4} M, which concentration was shown by

Lockhart and Potter (21) to be sufficient to block completely the activity of cytochrome oxidase. Was this the sole site of cyanide activity? The above-mentioned workers showed that, at a concentration of 10^{-2} M cyanide, the reduction of cytochrome c itself was inhibited, and they concluded that it is not unlikely that even 10^{-3} M cyanide has some depressant action on the reduction of cytochrome c. That 10^{-2} M cyanide does not inhibit diaphorase was reported by Adler *et al.* (1) and was later verified by Lockhart and Potter (21). Meyerhof and co-workers in 1938 reported that DPN itself reacts with cyanide (22). The investigations of Colowick and associates (4) not only confirmed the conclusions that the oxidized form of DPN is capable of forming a complex with cyanide but also presented evidence that reduced DPN is unaffected by cyanide. Hence, the locus of action of cyanide in the coenzyme I oxidase system seems primarily to be the terminal part of the oxidative chain rather than essential interaction with the components of the initial diaphorase system.

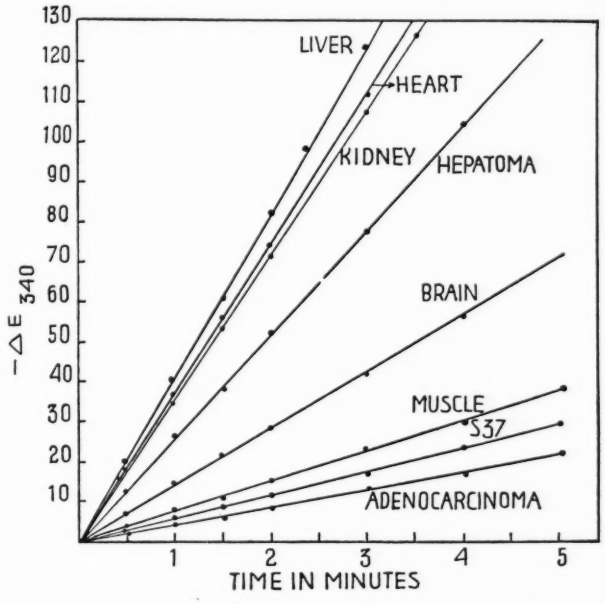

CHART 2.—The diaphorase activity of normal and tumor tissues of mice. The reaction mixture contained 0.3 cc. of 2×10^{-3} M KCN and 0.1 cc. of 5.3×10^{-4} M methylene blue. $-\Delta E_{340}$ values represent the decrease in optical densities $\times 1{,}000$.

In the diaphorase determinations (Chart 2) the sarcoma and adenocarcinoma showed the lowest activity, though this again approached closely the activity of normal muscle. The low activity of these tumors was not shared by hepatoma 98/15, which was superior in activity even to the brain, though significantly lower than the analogous normal liver. These data suggested that, in the case of the hepatoma, the low coenzyme oxidase response was not associated primarily with limited diaphorase activity but rather pointed to a failure of the cytochrome components. In the case of the other tumors, however, low diaphorase activity, together with a reduced ability to complete the normal oxidative pathway through the cytochromes, may be essentially contributory to the inefficiency of the total system.

Direct measurements of cytochrome oxidase activity by the manometric method of Schneider and Potter (29) confirmed the supposition of a deficiency in the cytochrome system for both hepatoma and the other tumors analyzed (Chart 3). These results agree with the recent observations of Schneider and Hogeboom (28), who found a decrease in cytochrome oxidase in homogenates of hepatoma 98/15 as compared to that in normal liver. The earlier reports of Greenstein (8) and other investigators (30, 31, 36) likewise lend supporting evidence to the conclusion that cytochrome oxidase activity is lowered in neoplastic tissues.

Additional information on the failure of the cytochrome system in the tumor extracts used in the present study was afforded by spectrophotometric experiments in which the normal coenzyme I oxidase system was supplemented with either limited or excess amounts of cytochrome c. Chart 4 shows the increase in optical density at 550 mμ ($+\Delta E_{550}$) in systems where the cytochrome oxidase is not inhibited. Cytochrome c, in these experiments, was used at a final concentration of 4.5×10^{-5}. The broad maximum clearly discernible in the curve for hepatoma indicates not only a somewhat delayed reduction of cytochrome c but

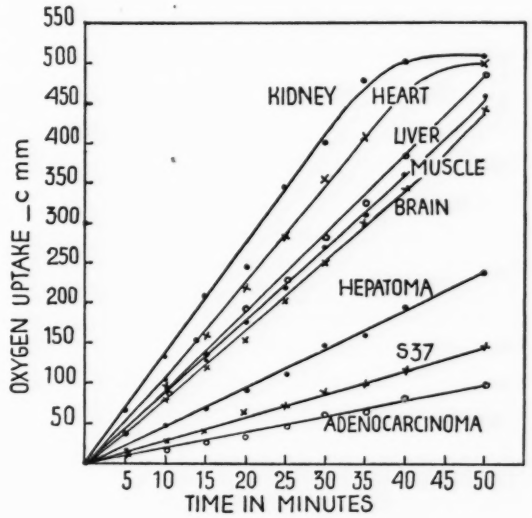

CHART 3.—The cytochrome oxidase activity of normal and tumor tissues of mice.

also a decreased cytochrome oxidase activity. The slope of the descending curve is due to cytochrome oxidase, the activity of which is not manifest until the oxidation of $DPNH_2$ is near completion or has actually terminated. Unlike the heart, kidney, and liver where the oxidation of $DPNH_2$ is completed very rapidly (Chart 5), hepatoma exhibited a falling off of the curve of absorption at 550 mμ only after approximately 3–4 minutes (Chart 4). The maximum of the curve also suggests the relative inefficiency of cytochrome oxidase for hepatoma. This can be deduced from the consideration that

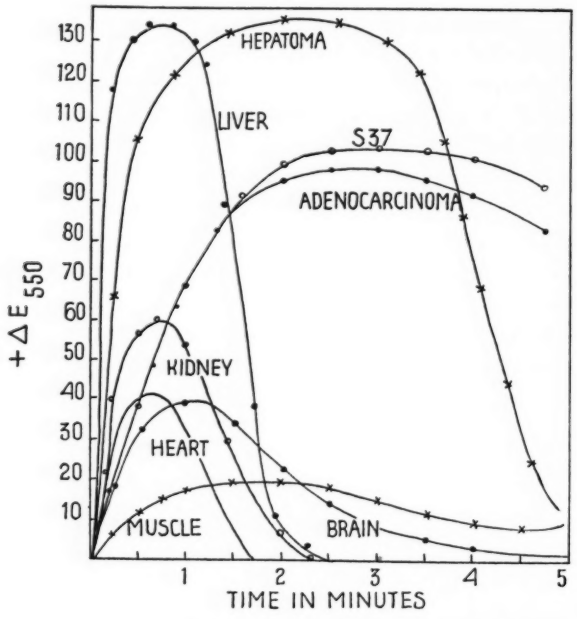

CHART 4.—The effect on the absorption at 550 mμ produced by the addition of 4.5×10^{-5} M cytochrome c to the coenzyme I oxidase system of normal and tumor tissues of mice. $+\Delta E_{550}$ values represent the increase in optical densities ×1,000.

the maximum attained would be of a lower magnitude were the cytochrome oxidase more efficient than the reductase system. Such a phenomenon is illustrated by the heart extract. Further interpretation of the activity of hepatoma as represented in Chart 4 should be made in light of the relatively good diaphorase activity (Chart 2) and markedly superior cytochrome c reductase activity (Chart 7) exhibited under other experimental conditions.

With regard to the other tumors, Chart 4 suggests a low oxidase activity for sarcoma and adenocarcinoma. Here, the cytochrome c reductase activity is decidedly inferior to that of the hepatoma (Chart 7). Before concluding from the data in Chart 4 that the sarcoma and adenocarcinoma have a lower cytochrome oxidase activity than hepatoma, it should be remembered that the oxidation of $DPNH_2$ was still in progress (Chart 5) when the extinction at 550 mμ began to decrease. However, a somewhat greater cytochrome oxidase activity for the hepatoma is indicated directly by the manometric results (Chart 3). (It should be noted here that the enzyme preparations used in the manometric studies were 30 times as concentrated as in the spectrophotometric experiments where enzyme solutions could be used at very high dilutions with reproducible results.)

That the metabolism of $DPNH_2$ normally requires cytochrome oxidase is now generally accepted. Recently, Slater (34) has demonstrated that 5×10^{-4} M KCN produced at 96.9 per cent inhibition of the coenzyme I oxidase system.

In addition, Slater observed that such inhibitors as azide, hydroxylamine, sulfide, and fluoride also depressed the coenzyme I oxidase system of heart muscle preparation. The present study confirms the inhibition of this system by several kinds of respiratory depressants. The effect of the following depressants on the heart, kidney, and liver preparations was examined: azide and hydroxylamine in final concentrations of

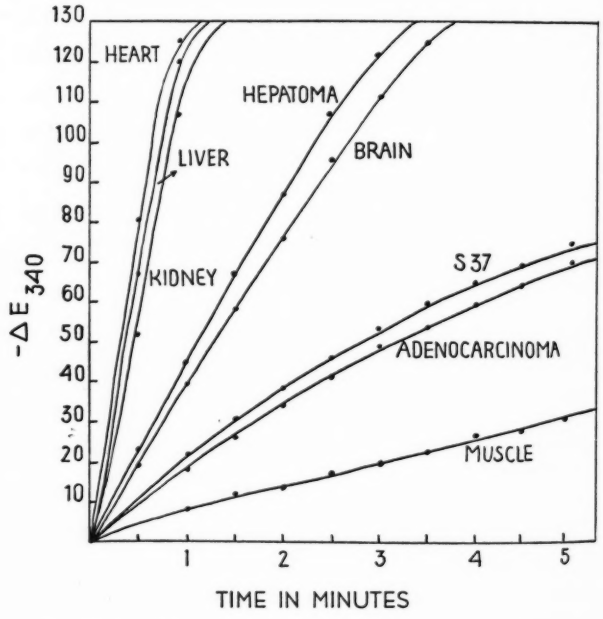

CHART 5.—The effect on the absorption at 340 mμ produced by the addition of 4.5×10^{-5} M cytochrome c to the coenzyme I oxidase system of normal and tumor tissues of mice. $-\Delta E_{340}$ values represent the decrease in optical densities. ×1,000.

1.6×10^{-2} M, *p*-chloromercuribenzoate, 1.6×10^{-4} M, and phenyl mercuric nitrate, 3×10^{-5} M. An appreciable inhibition of coenzyme I oxidase activity was observed in all experiments.

In determining the site of action of these depressants, the nature of the inhibitors should be borne in mind. *p*-Chloromercuribenzoate is known to react readily with sulfhydryl groups. However, Barron and Singer (3) reported that heart flavoprotein does not contain essential -SH groups. Nor, according to these authors, is cytochrome oxidase -SH dependent. It is probable that this mercurial as well as phenyl mercuric nitrate depresses not by formation of reversible mercaptides

with essential -SH groups but by virtue of the general property of mercurials to act with proteins, possibly by combination with the peptide groups of proteins or with the N of the imidazole group. This was demonstrated by Haarman (9) in the reaction between inorganic Hg and proteins. With reference to cytochrome oxidase, Kreke and co-workers (18) have obtained depression of activity through the use of either of the above-mentioned mercurials. Slater (33) also found that the oxidation of cytochrome c was inhibited by *p*-chloromercuribenzoate. It seems altogether likely, therefore, that the coenzyme I oxidase system is inhibited because of inactivation of cytochrome oxidase and, possibly, the factors mediating the reductase portion of the chain. The latter supposition is included, since the existence of heme or other metallic components in this part of the system has been postulated. These, accordingly, would be susceptible to inhibition by certain of the depressants tested. Conclusions concerning diaphorase inhibition cannot be made with any degree of certainty.

The formation of hydrogen peroxide in the oxidation of reduced yellow enzyme has been reported (25). It was of interest, therefore, to examine the effect produced by the addition of catalase to the coenzyme I oxidase system. An acceleration of activity was anticipated in view of the fact that peroxide is toxic to a number of sensitive enzymes. Contrary to expectations, however, a decrease in oxidative activity was observed (Table 2). A related phenomenon reported by Altschul *et al.* (2) may afford a likely explanation for this reaction. These workers found that catalase at a concentration of 1 μg/cc was sufficient to inhibit completely the activity of soluble cytochrome oxidase. Inactivation of this enzyme in the coenzyme I oxidase system is, therefore, highly probable.

Cytochrome c.—Concerning cytochrome c content of tumors, values lower than normal have been reported by a number of investigators (6, 8, 36). Rosenthal and Drabkin (27) reported a lowered cytochrome c content in rat hepatoma compared to normal liver. Indirect evidence obtained in the present study supports the conclusion that cytochrome c is diminished in malignant tissues. This evidence was obtained by supplementing the coenzyme I oxidase system of normal and tumor tissue extracts with cytochrome c and determining the degree of stimulation of oxidase activity thus elicited. In the presence of cytochrome c (Chart 5), a tremendous acceleration of coenzyme I oxidase was exhibited by the tumors. Hepatoma 98/15 was stimulated 30-fold; the other tumors, approximately 15-fold. The normal tissues, on the other hand, gave a much lower response. The oxidative rates for kidney and brain were accelerated 6 times; that for liver, 10 times. A relatively adequate amount of endogenous cytochrome c must be assumed in the case of the heart, since added cytochrome c increased the rate of oxidation of $DPNH_2$ only 3-fold. However, a high cytochrome c concentration in the tissue extracts prepared as described in this study is not to be expected even for heart, since the fractionation procedures employed were designed to remove certain soluble tissue metabolites. The catalytic effect of cytochrome c on the coenzyme I oxidase activity was reported also by Slater (34) who obtained an 11-fold increase in activity when cytochrome c was added to kidney preparation. A similar stimulation by cytochrome c of enzymes requiring coenzyme had previously been observed by other investigators (11, 21, 24).

Attempts to obtain a general notion of the actual cytochrome c content in the tissue extracts, normal and tumor, were made by measuring the increase in absorption at 550 mμ before and after the addition of small quantities of sodium dithionite. A low cytochrome c value was indicated, notably in the case of tumors, all of which had $+\Delta E_{550}$ values of 0.003–0.004. Heart, liver, and kidney gave values of 0.010–0.015. Obviously, such measurement yielded information on the concentrations of all tissues constituents, cytochrome c included, which show an increase in absorption at this wave length when treated with dithionite. Though not definitive, these data can be used in a negative manner, and it was found that, as such, they lent confirmatory evidence to the conclusions drawn from decisive experiments.

TABLE 2

THE INHIBITION OF COENZYME I OXIDASE BY CATALASE*

($-\Delta E_{340}$ represents the decrease in optical densities × 1,000)

	$-\Delta E_{340}$ Control	$-\Delta E_{340}$ Control+catalase
Muscle	9	3
Heart	134	90
Kidney	120	70
Brain	55	35
Adenocarcinoma	4	2

* $DPNH_2$ was prepared by nonenzymatic reduction.

It should be emphasized here that, in analyzing the systems supplemented with cytochrome c, an absolute correlation between the degree of acceleration obtained and the original cytochrome c content cannot be made with certitude for all the tissues, since a number of factors directly influence the activity of the whole respiratory system. However, collectively the data strongly indicate a diminished cytochrome c concentration in the case of the hepatoma. This is not the sole factor responsible for negligible coenzyme I oxidase activity, since the addition of considerable amounts of cytochrome c, 1.6×10^{-4} M, did not give oxidative

rates equal to those of the analogous liver or of the heart and kidney. Lehninger states (19) that 1.5×10^{-5} M cytochrome c is a saturating concentration for optimum activity of the succinoxidase system of rat liver.

Additional information on the specific activities of the components of the coenzyme I oxidase system was obtained by analyzing systems containing excess methylene blue (without cyanide) to facilitate the oxidation of reduced diaphorase. The acceleration of oxidase activity produced by added methylene blue is seen in Chart 6. The stimulation

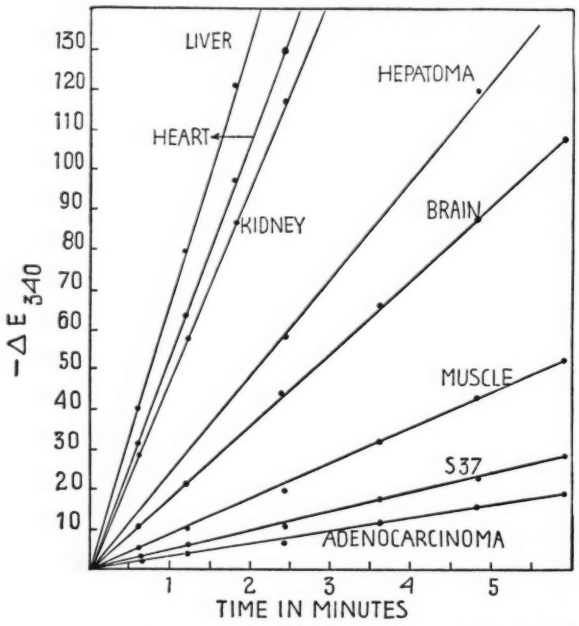

CHART 6.—The effect of 1.7×10^{-5} M methylene blue on the coenzyme I oxidase activity of normal and tumor tissues of mice. $-\Delta E_{340}$ values represent the decrease in optical densities $\times 1{,}000$.

obtained is less than that observed with cytochrome c, but the molar concentration of the latter was somewhat higher. The results of Chart 6 again suggest a decreased diaphorase activity for hepatoma, as compared to liver, and would seem to provide some degree of support to the conclusion already deduced from more direct data, that the cytochrome system is not exclusively limiting in the coenzyme I oxidase activity of hepatoma.

Cytochrome c reductase.—Cytochrome c reductase determinations (Chart 7) revealed greatest and almost identical activities for liver and hepatoma. Hogeboom and Schneider (13), in their studies of particulate and nonparticulate fractions of liver and hepatoma 98/15, observed an actual increase in the activity of this enzyme in hepatoma mitochondria. The finding that cytochrome c reductase is not lowered in hepatoma is especially striking in view of the fact that the diaphorase activity (Chart 2) is lower than that of normal liver. It seems likely, therefore, that, while there is a decreased efficiency of the diaphorase system, a failure of the one or several components constituting the factor which mediates the system between diaphorase and cytochrome c does not occur. Adequate concentrations of these factors can be surmised also for heart, liver, and kidney. Unfortunately, knowledge of the nature and mechanism of action of the intermediates in this link is meager. A number of studies have implicated cytochrome b as an intermediate (5, 14, 24), but the recent work of Slater (34) has demonstrated its nonparticipation. Slater believes his BAL-sensitive factor, which operates between cytochrome b and cytochrome c in the succinoxidase system, also functions in the coenzyme I oxidase system in the position between diaphorase and cytochrome c. In support of this belief, Slater found that 0.01 M BAL inactivated the coenzyme oxidase system 94.6 per cent (35). Lehninger's finding (19) that

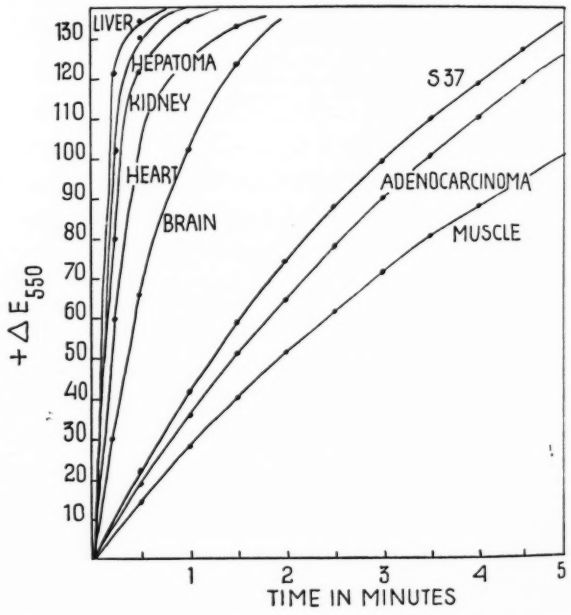

CHART 7.—The cytochrome c reductase activity of normal and tumor tissues of mice. $+\Delta E_{550}$ values represent the increase in optical densities $\times 1{,}000$.

phosphorylation is coupled to the oxidation of $DPNH_2$ may help considerably in elucidating the nature of the participants and their mode of action as intermediates in this respiratory system.

It is perhaps worthy of mention that an alternative explanation for high cytochrome c reductase and low diaphorase activity for hepatoma might be associated with the catalytic acceleration of coenzyme I oxidase activity by cytochrome c. In the former experiments the systems were supplemented with cytochrome c, while, in the latter, the

determinations were made without added quantities of this carrier. Whatever the explanation, it seems fairly reasonable to assume from the data available that, in the case of hepatoma, the unit linking the removal of hydrogen from diaphorase with the electron transfer to cytochrome c is not critically diminished.

In evaluating the cytochrome c reductase activity of the other tumors analyzed in this study, a moderately good degree of activity is indicated. This is suggested not only by Chart 7 but also by the slopes of the respective curves in Chart 4. It is to be recalled, however, that diaphorase values were low (as was also cytochrome oxidase). It would not be improbable, therefore, that the deficiency in the operation of the cytochrome c reductase unit was due more to the failure of the first step in the oxidation of $DPNH_2$ than to a diminished oxidative capacity of the reduced diaphorase cytochrome link.

It is of interest that, in the study of cytochrome c reductase systems containing cyanide, the authors were unable to obtain the maximum values for reduced cytochrome c when solid dithionite was added to the absorption cells after the experimental determinations were completed. In systems lacking cyanide, such was not the case. This suggested the formation of a cytochrome-cyanide complex which prevents reduction by dithionite. Indeed, Potter (26) observed that cytochrome c combines with cyanide in such a way that it cannot be reduced enzymatically. In the present study reduction was attempted by nonenzymatic methods. The formation of a cytochrome-cyanide complex has been further described by Horecker and Kornberg (14).

In reviewing the enzyme pattern for the normal tissues and in correlating this information with the data afforded by Chart 4, the following can be noted:

1. Liver manifests a marked ability to reduce cytochrome c, as evidenced in Chart 4 by the sharp rise of the curve to its maximum. The value of the maximum indicates that this system operates more efficiently than the cytochrome oxidase portion of the chain. This confirms the conclusions drawn from actual cytochrome c reductase determinations (Chart 7). A lower cytochrome c content for liver and kidney than for heart is suggested, since, given additional cytochrome c, the total coenzyme I oxidase activity of the first two tissues compares favorably with that of the heart. This is especially evident when it is recalled that the diaphorase and cytochrome c reductase systems are optimum for liver and closely approximated by kidney. The methylene blue experiments of Chart 6 supply additional information substantiating the same point.

2. With regard to the kidney and heart, the high cytochrome oxidase activity (Chart 3) explains the low maxima of the curves for these tissues. The appreciable cytochrome c reductase activity determines the time interval at which the system attains this maximum. For these two tissues, the very rapid oxidation of $DPNH_2$ is clearly shown by complete expenditure of the substrate in less than a minute (Chart 5).

3. A moderate diaphorase, cytochrome c reductase, and cytochrome oxidase activity is displayed by brain extract. The intermediate activity of cytochrome oxidase is also suggested to some degree by the slope of the descending curve in Chart 4, though, as was true of the tumors, the oxidation of $DPNH_2$ was still operative at this time and hence, too, the reduction of cytochrome c.

4. The low enzymatic activity of muscle tissue manifested by specific diaphorase and cytochrome c reductase determinations is further evidenced in Charts 4 and 5. Thus, cytochrome oxidase excepted, muscle shows greatly diminished concentrations of all of the components of the coenzyme I oxidase system.

The results of Charts 4 and 5 are of further significance in illustrating the direct linking of $DPNH_2$ oxidation with cytochrome c reduction. By making simultaneous determinations of de-

TABLE 3

GENERAL SUMMARY OF THE COENZYME I OXIDASE SYSTEM AND ITS COMPONENTS IN NORMAL AND TUMOR TISSUE EXTRACTS

	Coenzyme I oxidase	Diaphorase	Cytochrome c reductase	Cytochrome c	Cytochrome oxidase
Liver	+++	++++	+++++	++	+++
Kidney	++++	++++	++++	+++	++++
Heart	+++++	++++	++++	++++	++++
Brain	++	++	+++	++?	+++
Muscle	+	+	+	++?	+++
S-37	+	+	++	+	+
Adenocarcinoma	+	+	++	+	+
Hepatoma 98/15	+	+++	+++++	+	++

crease in optical density at 340 mμ with increase in optical density at 550 mμ, the close interdependence of these two systems was clearly established. In systems lacking cyanide but containing added cytochrome c, the over-all reactions were, in general, limited by the first part of the respiratory chain, while systems containing cyanide illustrated the importance of cytochrome c content.

In co-ordinating the results of this study, the enzyme patterns for the individual tissue extracts can perhaps best be summarized by representing a generalized picture of the degree of functioning of the known components of the coenzyme I oxidase system (Table 3). Conclusions as to the relative efficiency of each component are drawn from both direct and indirect information provided by the several lines of experimental approach.

SUMMARY

A comparative quantitative study of the ability of an acid-treated extract of various normal and tumor tissues of mice to oxidize $DPNH_2$ is presented. This oxidative system was analyzed quantitatively for the activity of its known constitutive enzymes: viz., diaphorase, cytochrome c reductase, and cytochrome oxidase. The responses of these units, together with those of cytochrome c and unknown factors mediating the system between reduced diaphorase and cytochrome c, are correlated to explain the over-all oxidative reactions obtained in the analysis of five normal tissues and several kinds of tumors. The impaired functioning of the complete coenzyme I oxidase system as displayed by hepatoma 98/15, when compared to normal mouse liver, is to be imputed chiefly to a failure of the cytochrome components of the respiratory chain, cytochrome c and cytochrome oxidase, though a somewhat diminished diaphorase activity has been demonstrated. The cytochrome c reductase activity was not lowered in hepatoma. A limiting activity of the factors mediating the oxidation of reduced diaphorase and the reduction of cytochrome c was likewise not indicated. The very low total oxidative reaction exhibited by the transplanted sarcomas and adenocarcinoma was shown to be due to a deficiency of a number of components, notably cytochrome c, cytochrome oxidase, and diaphorase. The cytochrome c reductase values were superior only to the muscle. The effect of various heavy metal and sulfhydryl depressants on the coenzyme I oxidase system was noted. A depression of the oxidative capacity was observed upon the addition of catalase. Evidence confirming the reaction between cytochrome c and cyanide is also presented by these studies.

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Further Observations on the Survival Time of Mice Bearing Chemically Induced Fibrosarcomas*

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Recent data have shown that the survival time of mice of the Prunt inbred strain bearing chemically induced fibrosarcomas is influenced by litter seriation (2, 3). It was determined that female mice developing fibrosarcomas with latent periods of less than 100 days lived longer when they belonged to late rather than early litters of the same breeding mice (188 mice) (2). This shift in survival time was not observed in female mice which developed tumors with latent periods between 101 and 200 days (148 mice) (3).

TABLE 1

EFFECT OF LITTER SERIATION ON SURVIVAL TIME OF MICE BEARING METHYLCHOLANTHRENE-INDUCED SARCOMAS

LATENT PERIOD	LITTERS	NO. MICE	SURVIVAL TIME Av.	SURVIVAL TIME Median	× P.E.
<100	1–3	74	69.3	66.0±2.5	
	4–11	131	92.8	99.0±2.4	
Difference			23.5	33.0±3.41	9.6
101–200	1–3	156	72.7	72.0±2.0	
	4–11	195	70.6	69.0±1.5	
Difference			2.1	3.0±2.5	1.2
Differences					
<100	1–3				
101–200	1–3		3.4	6.0±3.2	1.8
<100	4–11				
101–200	4–11		22.2	30.0±2.8	10.7
Total	1–11	556			

To date, similar observations on survival times with chemically induced fibrosarcomas on 556 female mice of the Prunt strain have been made. Of these, 205 developed fibrosarcomas with latent periods of less than 100 days, and 351 developed similar tumors between 101 and 200 days following the subcutaneous injection of 1 mg. of methylcholanthrene dissolved in 0.1 cc. of sesame oil at 60 days of age. These data include the data of the two previous studies on survival time (2, 3). Survival time is determined by the time between the appearance of a definite progressively growing tumor at the site of the injection of methylcholanthrene and the death of the animal. All mice showing nodules which regressed following their appearance are excluded from this analysis. All mice included in the study had tumors at the time of death. Even though the tumor may not have been the exclusive cause of death, it must have contributed to that end. Sections of all tumors were examined histologically.

The data are given in Table 1 and graphically in Chart 1. The number of mice in the late litters (beyond the fifth) is still small. Consequently, in order to make a statistical analysis of trends, data for mice of early litters (the first three) and of later litters (4–11) are grouped together and comparisons made between these two classes for each of two groups. One group consisted of mice developing fibrosarcomas with latent periods of less than 100 days, and the second group of mice with tumors with latent periods of between 101 and 200 days. Seventy-four mice belonging to litters 1–3, with chemically induced fibrosarcomas of latent periods less than 100 days, lived an average of 69.3 days. This gave a median value of 66.0 ± 2.5 days. The formula for probable error,

$$\text{P.E.}_{\text{median}} = \frac{\text{average quartile deviation from median}}{\sqrt{n}}$$

(where n is number of mice), was used to test levels of significance. One hundred and thirty-one female mice belonging to litters 4–11 within the same latent period class (latent period < 100 days) lived, on an average, 92.8 days. This is a median value of 99.0 ± 2.4 days. There is a difference of 33.0 ± 3.4 days between the two groups. This is 9.6 × P.E. Thus, there is a significant increase of survival time with female mice belonging to litters 4–11, as compared to female mice belonging to litters 1–3.

In the two groups of female mice developing fibrosarcomas with latent periods between 101 and 200 days, the following values on survival times were obtained: 156 female mice belonging to litters 1–3 showed an average survival time value of 72.7

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days. This is a median value of 72.0 ± 2.0 days. In the group belonging to litters 4–11, 195 female mice had average survival time values of 70.6 days. This gave a median value of 69.0 ± 1.5 days. The difference between the two groups 72.0 ± 2.0 − 69.0 ± 1.5 is only 3.0 ± 2.5 days, or 1.2 × P.E., which is not significant.

The difference between the two groups of female mice belonging to litters 1–3 (230 mice) is 72.0 ± 2.0 − 66.0 ± 2.5 days or 6.0 ± 3.2 days which is 1.8 × P.E. However, the difference between the two groups of female mice belonging to litters 4–11 (326 mice) is 99.0 ± 2.4 − 69.0 ± 1.5 or 30.0 ± 2.8 days. This is 10.7 × P.E., which is significant.

The present data provide further evidence on the previous conclusion based on smaller numbers that the survival time of female mice bearing chemically induced fibrosarcomas is significantly increased in successive litters—provided the latent period for the appearance of the fibrosarcomas is less than 100 days (2, 3). Why this increased survival time is present in mice of successive litters for the early appearing tumors and not for mice with latent periods between 101 and 200 days is not clear from the available data.

It has been shown in other studies of chemically induced fibrosarcomas (4, 5) that the number of mice developing fibrosarcomas with invasion into the body cavity is gradually reduced in mice of successive litters. It is now clear that this reduction in the invasiveness of fibrosarcomas with litter seriation is not due to changes in the survival time of mice—the mice actually live longer in successive litters with tumors of latent periods of less than 100 days and live as long in successive litters when the latent periods of chemically induced fibrosarcomas is between 101 and 200 days.

Female mice of advanced litters (4–11) differ more in survival time values of chemically induced fibrosarcomas than do female mice belonging to early litters (1–3) (medial difference, 30.0 ± 2.8 days, compared to 6.0 ± 3.2 days). This divergence in a biological characteristic is perhaps indicative of a fundamental biologic function whose nature is not yet indicated.

Shimkin (1) reported in 1941 that the mean length of survival of C3H and L strain mice with induced subcutaneous sarcomas was 4.36 ± 0.10 weeks, irrespective of whether the palpable tumors appeared early or late following the injection of the carcinogen. The present data do not confirm this conclusion.

SUMMARY

To date, 556 female mice of the Prunt inbred strain (*a*) have received subcutaneous injections, at 60 days of age, of 1 mg. of methylcholanthrene dissolved in 0.1 cc. of sesame oil; (*b*) have developed fibrosarcomas at the site of the injection, and (*c*) have died. The present study deals with observations on survival time, the time between the appearance of the fibrosarcomas and the death of the mice. Of these mice, 205 developed fibrosarcomas with latent periods of less than 100 days, and 351 with latent periods of between 101 and 200 days. Mice belonging to late litters live longer with chemically induced fibrosarcomas than mice of early litters, provided that the tumors have a latent period of less than 100 days. This increase does not occur when the latent period of tumors is between 101 and 200 days. Thus, female mice of advanced litters (4–11) differ more in survival time values of chemically induced fibrosarcomas than do female mice belonging to early litters (1–3). This divergence in a biological characteristic is indicative of a fundamental biologic function the nature of which is not yet indicated but perhaps may be included under the phenomenon of "tissue specificity."

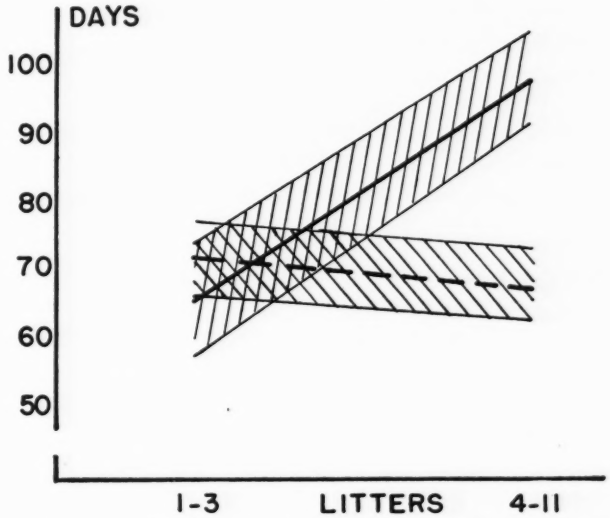

CHART 1.—Two groups of mice based upon litters 1–3 and 4–11 are given on the abscissa. Number of days for survival time (median) are plotted on the ordinate; data for mice with latent periods of less than 100 days, on solid line; comparable data for mice with tumors between 101 and 200 days, on the broken line. Shaded areas indicate 3 times Probable Error deviation from median.

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Tumor-inducing Properties of the Mammary Tumor Agent in Young and Adult Mice*

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Soon after the mammary tumor agent was found to be a causative factor in the genesis of mammary cancer in mice (9), it was determined that the agent could be transferred by grafts of normal tissues (13–14) from young mice of cancerous stocks and by the injection of extracts of either normal mammary glands (15) or mammary tumors (5, 17, 26). However, when mice which had been free of the agent obtained it by inoculation of an extract after they became adults, a low incidence of mammary tumors was observed (1, 4, 18, 21, 35). In 1945, Dmochowski (29) reported that, when 16-week-old BAF_1 hybrids received weekly injections containing large amounts of desiccated tumor tissue, the "age resistance" of the mice to the development of mammary cancer could be overcome. Each animal received by subcutaneous injection the amount of the agent from 1.5 gm. of dried tissue.

TABLE 1

OBSERVATIONS ON THE DEVELOPMENT OF MAMMARY CANCER IN ZBC FEMALES, INJECTED AT DIFFERENT AGES AND WITH VARIOUS AMOUNTS OF EXTRACTS OF TRANSPLANTED MAMMARY CANCER, CONTAINING THE MAMMARY TUMOR AGENT

Gm. equiv. per injection	No. of injections	Age in days when injected	No.	Cancer (per cent)	Av. age in days cancer	Av. age in days nonca.
2×10^{-2}	1	105–126	95	11.6	456	554
2×10^{-2}	1	72– 81	39	7.7	358	567
2×10^{-2}	1	57– 60	67	28.4	430	612
2×10^{-2}	1	44– 46	32	43.8	415	589
2×10^{-2}	1	23	67	64.2	385	444
10^{-3}	1	102–126	127	19.7	460	582
10^{-3}	1	72– 74	28	39.3	507	532
10^{-3}	1	57– 59	65	30.8	423	628
10^{-3}	1	44– 46	30	53.3	414	619
10^{-3}	1	22– 27	102	58.8	384	463
2×10^{-2}	5	112–126	48	6.3	497	582
2×10^{-2}	5	87– 88	40	15.0	521	602
2×10^{-2}	5	72– 76	35	22.9	466	583
2×10^{-2}	5	44– 46	30	53.3	484	582
10^{-3}	5	114–126	39	12.8	477	528
10^{-3}	5	87– 88	36	16.7	394	605
10^{-3}	5	72– 74	30	10.0	564	548
10^{-3}	5	44– 49	38	47.4	467	581

In view of past reports (5–8, 22–25, 33) that mice from our colony would have a high incidence of mammary cancer following the injection of the amount of the mammary tumor agent from either 10^{-3} or 10^{-4} gm. equivalents of tissue, the same technic was used to assay extracts of transplanted mammary tumors in adult mice. Young animals were injected for control purposes in each experiment. Additional data will be presented on the transmission of the agent by the experimental mice.

MATERIALS AND METHODS

The transplanted tumors, when used as source material for the agent, had been grown in mice which themselves did not possess the agent. The tissue was macerated by means of a tissue press (28), ground with sand, and extracted with distilled water to make a 20 per cent suspension. This was spun for 10 minutes in a clinical centrifuge, at approximately 2,500 r.p.m., following which procedure the supernatant fluid was removed and recentrifuged for the same time. The final supernatant fluid was diluted so that the injection of 1 cc. of the extract contained the amount of the agent, based upon the weight of the original tissue, specified in the tables. The extracts were given intraperitoneally.

The test animals were Ax (fostered A), Zb (fostered Z or C3H), and their reciprocal F_1 hybrids, and in no group was the incidence of mammary cancer higher than 0.4 per cent. The ZBC hybrids were produced by mating the F_1 females ($AxZbF_1$ or $ZbAxF_1$) with males of the fostered Zb stock, except in a few instances where Z males had to be used. The incidence in ZBC mice, kept as breeders, was less than 2 per cent (23, 25). Details regarding the age of the mice at the time of the experiment, dose, etc., are given in the tables for the respective studies.

Experiment 1.—This series was started on 7/30/48, and all the experimental mice, with the exception of one group of young ZBC mice, were injected with an extract of mammary tumor which arose in a mouse of the Z or C3H stock. The tumor had been transplanted for seven passages. The mice that received other injections were inoculated on 7/31, 8/2, 8/3, and 8/4. Another group of young mice was tested with the extract used on 8/3 to make certain that it contained the agent. Whenever young mice were included, they were the last group to be inoculated with the respective fractions.

The results observed on the development of mammary cancer in the experimental animals are tabulated in Table 1 with

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the incidence, average age at the time of appearance of the tumors, average age at death of the noncancerous mice, etc. Only noncancerous mice which survived for longer than 300 days were included. The two groups of young mice, used to assay the extracts for the first and fourth injections of some mice, have been combined, since no significant difference was noted.

In general, the mice of the various age groups which received the amount of the agent from 10^{-3} gm. equivalents of the tumor showed not only the highest incidence but, with one exception, had the earliest average cancer age. Conversely, the lowest incidence and latest average cancer age was found, for mice of the oldest groups, in those having five injections of the extract with the highest concentration of the agent. The younger the mice were at the time of injection, the higher was the percentage of mammary tumors. This was evident when the data for the 22- to 27-day-old mice were compared to observations from older animals.

Experiment 2.—Preliminary data may be cited for another study where Ax, Zb, their reciprocal F_1 hybrids, and ZBC females, 133–153 days of age, received a single injection of an extract of another transplanted mammary tumor. This arose in an AZF_1 female and had been carried in agent-free mice for nine passages. Extraction of tumor AZF_1 No. 8415 was such that the test animals received the amount of the agent from 5×10^{-2}, 10^{-3}, or 10^{-4} gm. equivalents of tissue. Among 102 Ax, Zb, and the $AxZbF_1$ or $ZbAxF_1$ hybrids injected with the various fractions, six tumors have been observed after 20 months, while the incidence for the ZBC females (131 mice) had been 24 per cent (Table 3). However, females of the inbred stocks and the F_1 hybrids became infected because mammary tumors have deveioped in their progeny. In these crosses, only males without the agent were mated with the injected females.

Young ZBC females, 22–24 days of age, were tested with the same fractions to make certain that they contained the mammary tumor agent. To date, 120 (90 per cent of the total) have developed mammary tumors, at average ages from 328 to 345 days. Three mice are under observation in the control series.

Experiment 3.—Data were reported in 1948 (23) on the development of mammary cancer in young ZBC females following the injection of a tumor extract with preliminary observations on the progeny of the experimental mice. This experiment is now complete, and the results for another series may also be included, since they received the same fractions. The source tissues for the agent were spontaneous mammary cancers from breeding Z females.

It was again observed (Table 4) that the extract with the lowest concentration of the agent produced not only the high-

TABLE 2

OBSERVATIONS ON THE DEVELOPMENT OF MAMMARY CANCER IN PROGENY BORN TO ZBC FEMALES (MATED WITH D^8 MALES) WHICH OBTAINED THE AGENT WHEN 126 DAYS OF AGE (TABLE 1)

	Treatment of mothers		Born 1st litters		Observations for all progeny		
	Gm. equiv.	No. of		Cancer		Av. age in days	
Mothers	each dose	injections	Ca/nonca	age	Ca/nonca	Cancer	nonca.
2 ca. ♀	10^{-3}	1	3/0	377	15/0	351	
3 nonca.	10^{-3}	1	6/1	325	25/6	336	664
Total	10^{-3}	1	9/1	343	40/6	342	664
4 nonca.	2×10^{-2}	1	4/5	339	16/11	331	700
5 nonca.	2×10^{-2}	5	1/12	443	1/12	443	698

Fourteen females from three of the injected groups were mated with males of the susceptible D^8 line (23), and the observations for the progeny are recorded in Table 2. All mothers had been injected when they were 126 days of age and were mated the day after some had received their fifth injection.

Two of the mothers, which received the smallest amount of the agent, developed mammary cancer at an average age of 512 days, while the others died free of tumors when they were from 332 to 734 days of age (average, 676 days). The noncancerous females had their first litters from 41 to 51 days (average, 44 days) after their first injection, and the two cancerous females had their first litters on the 48th and 53d days. Only the first litters born to the five females which received five injections of the extract diluted 50-fold are available to give data for this group, as the male in the breeding pen died and was not replaced. For comparative purposes, the progeny born to females of the other series are listed according to their first litters and the total number of offspring. These nine females had from five to eight litters each.

As seen in Table 2, the offspring born in the first litters to females which had been injected with multiple doses of the more concentrated fraction showed the lowest incidence, while the offspring born to mothers receiving the smallest amount of the agent had the highest incidence. These females obtained a 100-fold difference in the amount of the agent. Based upon small numbers, where comparisons could be made, there was no significant difference in either the incidence or the average cancer age between mice born in the first litters and the total number of young. The three females which died noncancerous after they obtained the agent from 10^{-3} gm. equivalents of tissue had offspring with an incidence of 81 per cent at an average age of 335 days. Considering only the first litters born to all females of the three groups, as the concentration of the extracts administered to mothers increased, the incidence in their progeny decreased, to 90, 44, and 8 per cent, respectively.

TABLE 3

PRELIMINARY DATA OBTAINED FOLLOWING THE INJECTION OF DIFFERENT AMOUNTS OF THE SAME TUMOR EXTRACT INTO VARIOUS GROUPS OF MICE

The mice were 133–153 days of age at the time they were injected.

Test animals	Gm. equiv. injected	No. injected	No. cancer
Ax	10^{-4}	10	0
Zb	10^{-4}	10	0
F_1	10^{-4}	10	3
ZBC	10^{-4}	42	12
Ax	10^{-3}	10	0
Zb	10^{-3}	10	0
F_1	10^{-3}	10	0
ZBC	10^{-3}	43	9
Ax	5×10^{-2}	14	2
Zb	5×10^{-2}	12	0
F_1	5×10^{-2}	16	1
ZBC	5×10^{-2}	46	11

est incidence in the ZBC females, 23–25 days of age, but they had the shortest latent period before tumors appeared. A small number of AX females, 62–70 days of age, showed few tumors.

Some experimental females were mated with Z males, and the observations for their progeny have been tabulated in Table 5.

Whether the injected females had mammary cancer or not, all had some cancerous progeny. However, the numbers are too small to draw conclusions about incidence and age. For example, the five Ax females, injected when 66 days of age with a 10 per cent suspension, had progeny with the same incidence and average cancer age as did the four ZBC females that received the same extract when they were from 23 to 25 days of age.[1] Three of the four ZBC mothers had tumors, whereas the five Ax females did not.

TABLE 4

DEVELOPMENT OF MAMMARY CANCER IN ZBC AND AX FEMALES FOLLOWING THE INJECTION OF AN EXTRACT OF MAMMARY CANCER FROM THE Z STOCK

Test mice	Age in days when injected	Gm. equiv. injected	No.	Cancer (per cent)	Av. age in days Cancer	Nonca.
ZBC	23–25	10^{-1}	20	70	373	427
ZBC	23–25	10^{-2}	20	65	376	585
ZBC	23–25	10^{-3}	20	85	327	500
Ax	66–70	10^{-1}	9	22	373	450
Ax	62–64	10^{-3}	6	33	615	541

TABLE 5

OCCURRENCE OF MAMMARY CANCER IN THE PROGENY OF ZBC AND AX FEMALES FOLLOWING THE INJECTION OF AN EXTRACT OF MAMMARY TUMOR IN THE MOTHERS (TABLE 4)

Stock of mothers	Gm. equiv. injected	Diagnosis of mothers	Progeny born 1st litters Ca/nonca	Ca. age	Observations for all progeny Ca/nonca	Av. age in days Cancer	nonca.
ZBC	10^{-1}	3 cancer	4/0	347	8/2	329	267
ZBC	10^{-1}	1 nonca.	2/1	542	4/1	373	513
ZBC	10^{-1}	Total	6/1	412	12/3	343	349
ZBC	10^{-3}	2 cancer	2/0	313	16/1	287	270
ZBC	10^{-3}	3 nonca.	0/0		4/0	338	
ZBC	10^{-3}	Total	2/0	313	20/1	297	270
Ax	10^{-1}	5 nonca.	13/5	341	19/5	330	729
Ax	10^{-3}	3 nonca.	3/3	480	10/4	362	634

DISCUSSION

The mammary tumor agent is widely distributed in the tissues of mice of cancerous strains (7, 21, 22). Although the young do not become "infected" until after birth, the agent may be recovered from the placenta, where it is probably located in the maternal blood in small amounts (30–31). For biological studies on the agent, lactating mammary glands (21, 23) and spontaneous (6, 23) and transplanted (23) mammary tumors have been shown to be good sources of the agent; but it may be transferred by grafts of normal tissues (spleen, thymus, mammary glands) of young mice of cancerous strains (13).

[1] Two of the young listed as living in the previous report are not included, as they died before the youngest mouse had cancer.

In numerous investigations, it has been found that, following the administration of the agent to mice of different ages, adult mice become more resistant to the induction of mammary cancer with increasing age. It was suggested that possibly mammary tumors did not appear because the agent was needed before the mammary glands had developed (18). This theory was advanced, since others (36, 37) had previously reported that a difference in the architecture of the glands could be detected in mice of the same stock, with and without the mammary tumor agent. This morphological difference could not be found when females of other inbred strains and their hybrids were examined (34).

Andervont, Shimkin, and Bryan (4) showed that the oral administration of milk containing the agent produced few tumors in mice 3.5–4 months of age, whereas a high incidence was found when young animals were tested. They thought it was possible the agent either was destroyed or could not penetrate through the gastrointestinal tract of the older animals. Females which had been kept as either virgins or breeders for 270 days were also studied, and a low incidence was observed after the intraperitoneal injection of an extract with the agent (21). In another study, Andervont (1) used 4–5-month-old females which had had one litter, and the introduction of the agent into these adult animals produced tumors in two of 25.

Further interest in the problem was stimulated by the publication of Dmochowski (29), who showed that the repeated injection of the mammary tumor agent present in dried tumor tissue would induce mammary cancer in 4-month-old females which had previously littered. The amount of material obtained from 0.125 gm. of tissue was resuspended for each injection. Although four weekly injections did not cause a marked increase in the incidence, a high incidence was seen after twelve weekly inoculations. In the latter group,

the mice would obtain the amount of the agent from 1.5 gm. of tumor.

Dmochowski explained his results on the basis of a difference in the action of the agent and the hormones in the development of mammary cancer in mice. He suggested that, while the hormones may induce mammary cancer in susceptible mice only when the mammary glands have developed in the presence of the agent, the agent could exert its action in mice even after their glands had been stimulated by hormones, in the absence of the mammary tumor agent.

The data considered here would appear to refute the interpretation offered by Dmochowski in that we observed the highest incidence in the oldest mice following the administration of extracts with the lowest concentration of the agent. Several suggestions may be afforded to interpret the differences between the two studies.

By the use of supernatant fluids from centrifugation at low speeds of either normal or tumor tissues, the results for our colony have been quite consistent in that the amount of the agent obtained from 10^{-3}, or 10^{-4}, gm. equivalents of the tissue may be as active in producing mammary tumors as 10^{-1} gm. equivalents. In fresh tissue, there may be an inhibitor which might be diluted out faster than the agent, and the inhibitor could be destroyed during the process of desiccation. It is also probable that the amount of the agent present in the same amount of fresh tissue would be considerably less following desiccation. Thus, the injection of the agent from 1 gm. of dried tissue might be comparable to the use of a much smaller amount of fresh material. Whereas Dmochowski (29) gave twelve weekly injections, we used five injections over a period of 6 days.[2]

As indicated by one study in this report (Table 3), the introduction of the same fraction of a tumor need not induce the same incidence of mammary cancer in mice of different groups, even though the mice are closely related. Primary consideration must be given to possible differences in the genetic susceptibilities of the test animals, including end-organ sensitivity and hormonal mechanisms.

Males of the C3H stock, without the mammary tumor agent, have been used to test the association between the state of development of the mammary glands at the time of the administration of the mammary tumor agent and the later development of mammary cancer (35). The males that were castrated when 4 weeks old and grafted with ovaries from females of the same stock and age, followed by the injection of the agent the following day, gave an incidence of mammary tumors of 74 per cent. In other groups, the mice either were castrated and received the ovarian grafts at 1 month and the agent when 4 months of age, or were injected with the agent when 4 weeks of age and were castrated at 4 months when they also obtained implanted ovaries. In mice of these latter groups, the incidence was approximately 25 per cent, yet the average latent period of tumor development after the injection of the agent was approximately the same in all groups. This would suggest that the relative resistance of older mice may not be due to the degree of mammary development of the time of infection with the agent.

The use of repeated injections of large amounts of dried tumor tissue by Dmochowski (29) induced a high incidence of mammary cancer in females of the BAF_1 generation. When females of the reciprocal generation (ABF_1 hybrids) were maintained as virgins, a low incidence of mammary tumors was noted (12). The ABF_1 females would obtain the mammary tumor agent and the inherited susceptibility from their mothers of the cancerous A stock, but neither of the parental strains would transmit the inherited hormonal influence (27) associated with the development of mammary cancer in virgins. In the absence of this hormonal mechanism possessed by the Z or C3H stock, there may exist some difference in the mammary glands which would make them sensitive to the development of mammary cancer following the injection of the mammary tumor agent when they became adults.

The mammary tumor agent transferred by females of cancerous strains does not induce the same incidence of mammary cancer in hybrids with the same genetic constitutions (10). This subject has recently been reviewed (23), and the data would indicate that we may be dealing with different agents. To complicate the problem even more, extracts of spontaneous mammary tumors from females of the same cancerous inbred strains may not show the same activity when injected into comparable test animals (unpublished data). The factors that may be involved are not understood at this time.

In the first series of the foster-nursing studies which demonstrated the mammary tumor agent, it was observed that, while some of the fostered fe-

[2] Since the adult ZBC mice and their progeny which received five injections of the tumor extract diluted 50-fold had the lowest incidence, Z or C3H females, 5–8 weeks of age, were tested to determine if their incidence and average cancer age might be altered by subjecting them to the same treatment. The amount of the agent from either 10^{-1} or 10^{-3} gm. equivalents of transplanted tumors was administered for 5 successive days. No change has been noted in either the incidence or the average cancer age of the experimental mice and their progeny.

males died without mammary cancer, cancer appeared among their progeny and descendants (9, 11). Likewise, females of a low susceptible B (C57 black) line were found to have a low incidence after they were nursed by females of cancerous stocks; but, when these B females with the agent were used to nurse susceptible mice, these susceptible animals gave rise to mammary cancer (14). The observations demonstrated that infected mice of either susceptible or relatively nonsusceptible strains may die noncancerous, but they will transfer the agent to their progeny.

In other cases, the agent may "appear" in mice born of parents neither of which possessed the agent (16, 23). If progeny have been continued when this occurs, they give rise to a cancerous line (15), or the agent may be recovered from the mammary tumors that appear, as shown by biological assay (23, 25).

Evidence has accumulated that the transmission of the agent in the milk of females which obtained the agent by the grafting of normal tissues, the injection of an extract, or infection from the male, is comparable to that seen when the mice secure the agent by nursing. Following the grafting of normal tissues (spleen, thymus, mammary glands), even noncancerous females transferred the agent to their progeny (13, 14). Some of these experimental mice had had one litter before they had been inoculated with the normal tissue from young donors.

Andervont (1) used females from 4 to 5 months of age, after they had given birth to one litter; and, following the injection of a tumor extract, only two of 25 had mammary tumors. When the injected females were mated with males of the cancerous C3H stock, seven of the females had cancerous progeny, but the offspring born to fourteen other females remained free of mammary tumors. He concluded that, while the adult mice might be resistant to the agent, adults may become infected and pass the agent to their progeny.

In 1948 (23) data were reported on the development of a higher incidence in young mice which received the amount of the agent from 10^{-3} than from 10^{-1} gm. equivalents of tumor tissue, and the experimental females which obtained the smaller amount of the agent also had progeny with the higher incidence. Injected mice which died noncancerous but had cancerous progeny (Tables 4 and 5) were included.

It was observed in several experiments that mice developed mammary cancer following the administration of the agent in dilutions representing 10^{-6} (5, 23) or 10^{-7} (33) gm. of either normal mammary tissue or mammary cancer. Andervont (2) found that mice which obtain comparable amounts of the agent may have cancerous progeny, showing that they were infected.

When females of various stocks become infected with the agent when it is transferred by males of cancerous strains (3, 24, 25, 32), strain differences in the sensitivity of the females to infection, as well as the ability of the males to infect, may be seen by studying various crosses. Whereas Andervont and Dunn (3) found that less than 5 per cent of the C females developed mammary cancer when mated with males of the Andervont C3H stock, if males of our subline of the C3H strain were used, 59 per cent of the C females developed mammary cancer, and others became infected. The incidence in C females mated with males of the A strain was 33 per cent (24), and only 3 per cent of the C3H females without the agent gave rise to mammary cancer when crossed with males of the C3H cancerous line (19, 25). In our studies, the progeny born to females prior to infection from the male showed a low incidence; but, following infection, the progeny had a high incidence. Infection may not take place until the females have eight litters. It would be difficult to interpret the appearance of the high incidence in the C female and their progeny as resulting from the introduction of a large amount of the agent.

Further studies are indicated to determine the possible relationship between the induction of mammary cancer following the introduction of the mammary tumor agent into adult mice and the amount of the agent injected, genetic factors, possible hormonal mechanisms, the transmission of the agent, etc.

SUMMARY

The mammary tumor-inducing activity of the mammary tumor agent was studied by the administration of either one or five injections of fractions of transplanted mammary cancer into mice ranging in age from 22 to 126 days.

The concentration of the fractions represented the amount of the agent derived from either 2×10^{-2} or 10^{-3} gm. equivalents of fresh tissue.

In general, the incidences observed in adult mice could not be correlated with the amount of the agent that was injected.

The highest incidence occurred in the progeny of the mice which received a single injection of the fraction with the lowest concentration of the agent.

The transmission of the agent was observed following the administration of the mammary tumor agent into both young and adult females.

No significant difference was to be seen in the

transmission of the agent in the milk of either cancerous or noncancerous experimental mice.

Data were presented suggesting that the age resistance to the tumor-inducing action of the mammary tumor agent may be determined, in part, by the genetic constitution of the test animals. Other possibilities were discussed.

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Infrared Absorption Curves for Normal and Neoplastic Tissues and Related Biological Substances

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At the present time infrared investigations with biological materials are mainly empirical. This presentation will be no exception. Mathematical analyses of the vibrational modes of many molecules containing from 2 to 12 atoms have been summarized by G. Herzberg (8). With the increase in the number of atomic constituents, the analysis, generally, becomes more complex. Structural asymmetry has the same effect. Thus, complete theoretical discussions of molecules containing more than 12 atoms are few in number.

The complexity of the majority of the conglomerations designated as biological substances precludes, at the present time, theoretical prediction of the normal vibrations. However, certain molecular groups absorb infrared radiation within fairly well defined wave-length intervals—i.e., their absorption bands are fairly stable. This factor, along with others such as the empirical nature of the skeletal or "backbone" vibrations, often assists in partial determination of structure and chemical identification.

Infrared absorption curves of many components of the human body, such as various types of tissue, blood, plasma, and corpuscles, are quite similar in the region of 1–8 μ. This is to be expected, since the assignments in this region are those due to commonly occurring structure groups. As a result, the investigation of this spectral region may not be fruitful in the empirical infrared analysis of such proteins. In the region of 8–15 μ, where absorptions due to skeletal as well as certain structural vibrations generally occur, there are often noticeable differences.

The primary objective of this work was to investigate the infrared absorption of neoplastic and normal tissue for the spectral region 1–15 μ, with emphasis on the 8–15-μ interval.

Noteworthy progress along these lines has already been realized by E. Blout and his co-workers (1, 2). This important subject, however, warrants much further study.

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PROCEDURE

The experimental procedure in broad outline was to secure infrared data for (*a*) normal and neoplastic tissues, (*b*) nuclei and cytoplasm from normal and neoplastic tissues, and (*c*) important chemical constituents of cells.

Infrared data were taken on a manually operated Beckman infrared spectrophotometer (Model 270). All samples were either deposited or mounted on single silver chloride discs which served as supports. Some of the tissues were mashed by passing through a sieve under pressure. Others were microtomed with the use of the frozen-section technic. Solid materials in the crystalline or amorphous state were finely ground (9) and dissolved or suspended in water or absolute ethyl alcohol. After the materials were transferred to the silver chloride discs, they were slowly dried under an infrared lamp.

Particle size is a very important factor in determining the amount of scattering of radiation. Increased scattering in the case of solid organic chemicals, predominantly in the lower wave-length regions, is generally a consequence of an increase in particle size, of aggregation of particles, and of formation of nonuniform crystals while the sample is being dried. In this regard the choice of solvent or suspending medium is very important.

To reduce the scattering of radiation, it is sometimes helpful to mull powders or coat tissues with paraffin oil. This technic was tried but was not used in obtaining the final absorption curve data, because the absolute intensities of the absorption bands were not desired. Furthermore, this procedure generally requires that the sample be placed between two windows. Such an arrangement is analogous in some respects to a Fabry and Perot interferometer. At any rate, at certain separations of the discs, disturbing interference bands will appear in the spectrum. This is especially true when the absorption cell windows are silver chloride, since this material has high reflectivity. In addition, the C—H vibrations of the sample are masked by those of the paraffin oil.

In comparing data on infrared absorption for biological materials, it is of import to consider the manner in which the samples were prepared, for there is often a difference in the position and nature of absorption bands for substances, depending upon the physical state. Glassy films, though free from appreciable scattering, may yield absorption curves somewhat different from those of finely powdered samples. Thus, it is expedient, when feasible, to secure curves for the different physical states.

Drastic procedures for the production of films, such as sublimation, were not employed.

The separations of nuclei and cytoplasm mentioned above were accomplished by a citric acid method described by A. Marshak (13). The amount of citric acid added to the minced and strained tissue was substantially reduced, and the nuclei were rinsed several times with an isotonic solution of sodium chloride in the final centrifugations. These slight modifications

lessened the intensity of the interfering absorption bands of the citric acid. It should be pointed out that the chemical composition of the separated nuclei depends on pH (5, 6). For lower concentrations of citric acid, the nucleic acid content is often increased.

RESULTS AND DISCUSSION

Charts 1–12 are infrared absorption curves for various types of normal and neoplastic tissues. For the sake of completeness and for additional information about the amount of radiation scattered, the curves cover the spectral region of 1–8 μ as well as the region of greater interest, 8–15 μ. The possible empirical structural assignments for the former region are known for the majority of absorption bands and are treated in detail by Randall (14).

Since the materials under investigation are so complex, each absorption is probably a composite structure resulting from several different types of vibration.

The strongest bands for tissues generally occur between the wave-lengths 5.45 and 6.5 μ. This is the interval in which the carbonyl groups absorb. In the neighborhoods of 3.0 and 3.4 μ, the absorption is generally due to hydrogen stretching relative to nitrogen or carbon. Unassociated hydrogen stretching vibrations fall between 2.6 and 3.0 μ for O—H, 2.9 and 3.3 for N—H, and 3.0 and 3.7 for C—H.

There are other possible causes of absorption near 3.0 μ, such as vibrations due to the C—O group in cyclic diacylimides or urea and bending vibrations due to the NH_2 group in amines or true amides.

In tissues there is a fairly intense band near 6.5 μ. Mono-substituted amides absorb from 6.4 to 6.7 μ; amido acids, 6.4 to 6.6; and amino acids, 6.47 to 6.67. For the first type of compound the absorptions are attributed to the C—O and C—N groups, and for the last to NH_2 and CO_2^-. H. Lenormant (11, 12) attributes to the NH_3^+ group a band at 6.59 μ which is found in the absorption spectrum of many amino acids.

CH_2 and CH_3 bending vibrations fall in the region 6.65–7.6 μ. H. Lenormant (11) states that J. Lecomte attributes a band at 6.84 μ to a deformation vibration of the CH_2 group and one at 7.26 μ to a symmetric vibration of deformation of the CH_3 group. These assignments were made for amino acid spectra.

Above 8 μ the skeletal vibrations generally mask the bond type. In addition, the positions of the latter are less definite. In a great number of tissues there is a more or less pronounced band at about 8.1 μ. For a number of theoretically analyzed, simple molecules, absorptions in this vicinity have been assigned to rocking, wagging, deformation, bending, or twisting vibrations of CH, CH_2, or CH_3 structures. Kuhn (10) states that a strong band at 8.0–8.2 is characteristic of esters.

For some tissues there is often a broad pronounced band at approximately 9.2 μ. For others there are two or more bands in the region 8.8–10 μ. Definite bands are also observed occasionally in the region 10–11 μ, especially at about 10.2 μ. From 11 to 15 μ the curves are, as a rule, not very interesting.

A survey of the curves for neoplastic tissues shows that there is usually a characteristic absorption pattern in the region between 8.0 and 11.0 μ, involving absorption bands at approximately 8.1, 9.2, and 10.25 μ. This was found true for metastases of the liver and lung, a malignant lymph node of the neck, and the Marsh strain of mouse tumor. For the first two neoplasms the pattern was quite pronounced.

Charts 13–16 show that fat and blood and its components, serum and erythrocytes, do not exhibit the characteristic pattern mentioned above.

In order to secure more information about configurations causing absorption in complex tissues, infrared curves were determined for nuclei and cytoplasm separated from normal DBA mouse liver and from dbrB mammary tumor. The results are shown in Charts 17–26. With the same aim in mind, absorption data were obtained for important constituents of tissues and for some related compounds. These data are included in Charts 27–39.

Normal mouse liver and the light fraction of its cytoplasm show similar absorption patterns between 9 and 10 μ. In both there are bands at 9.2 and 9.65 μ. From Chart 22 it can be seen that citric acid, fortunately, does not exhibit any pronounced bands in this region. At lower wavelengths, however, its bands cause appreciable interference. It is interesting to note that citric acid has a strong band at 2.9 μ due to the OH group, while in mouse liver and a mixture of mouse liver and citric acid the nearest maximum absorption is 3.0 μ. The fact that the base of the curve is not broadened or that two absorption maxima, one at 2.9 μ and the other at 3.0, are not present may be due to association. This observation strengthens the point of view that it is generally not true that an absorption band such as the one with a maximum at 3.0 μ in a complex substance like mouse liver is due to a single assignment, namely, hydrogen stretching relative to nitrogen. The pattern for the heavy fraction of cytoplasm is different from

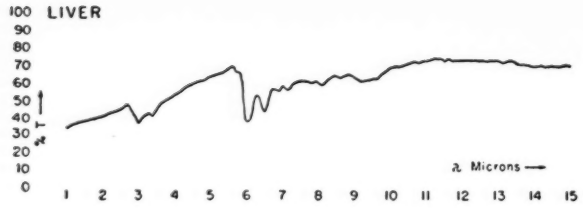

FIG. 1.—Normal liver tissue, mashed

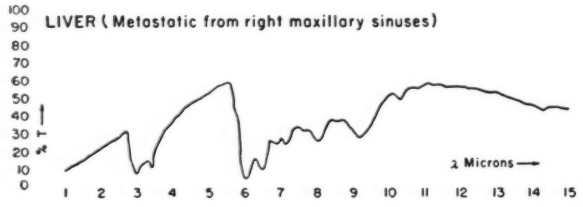

FIG. 2.—Metastasis in liver, mashed. Relatively thick sample. Note pronounced band pattern between 8 and 10.5 μ.

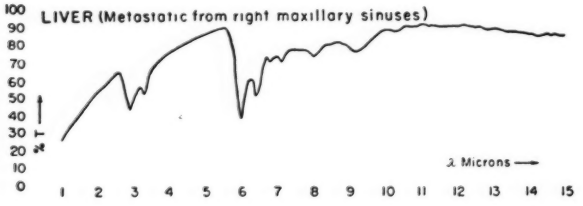

FIG. 3.—Metastasis in liver, mashed. Relatively thin sample.

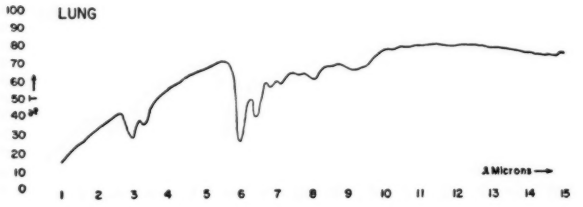

FIG. 4.—Normal lung tissue, mashed

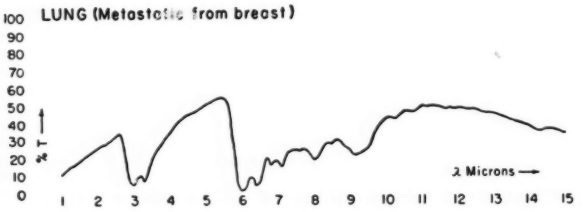

FIG. 5.—Metastasis in lung, mashed

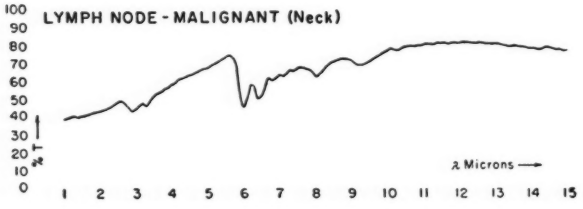

FIG. 6.—Malignant lymph node in neck, mashed

FIG. 7.—Normal spleen tissue, mashed

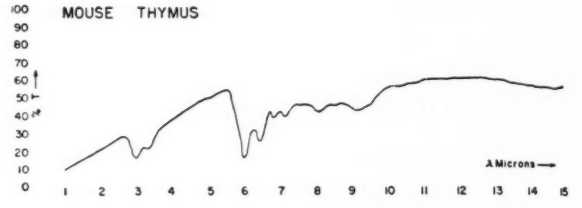

FIG. 8.—Normal thymus, mashed (from DBA mouse)

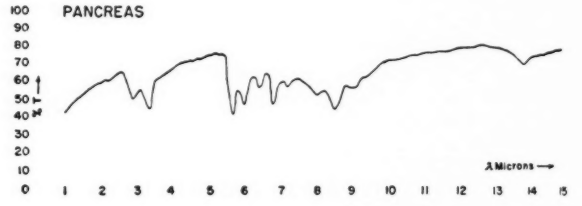

FIG. 9.—Normal pancreas tissue, mashed

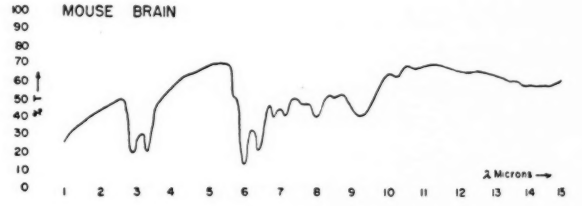

FIG. 10.—DBA mouse brain tissue, mashed

FIG. 11.—Tissue from aorta, microtomed. Thickness 10 μ

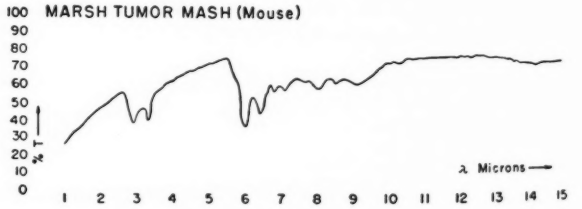

FIG. 12.—Marsh mouse tumor tissue

FIGS. 1–39.—Infrared absorption curves for tissues and related biological materials. Tissues are human unless otherwise noted. The symbols *W* and *A* connote that the sample was initially dissolved or suspended in water or alcohol respectively. The mouse tumor was dbrB mammary tumor unless otherwise noted. Per cent transmission of the sample is plotted along the ordinate versus wave-length in μ along the abscissa. The sample temperature was 26° C. during the infrared measurements.

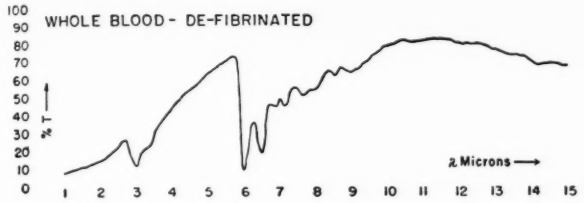

FIG. 13.—Defibrinated whole blood (human)

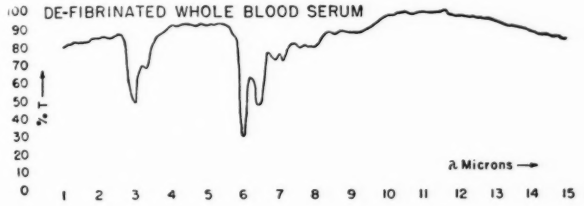

FIG. 14.—Blood serum (human)

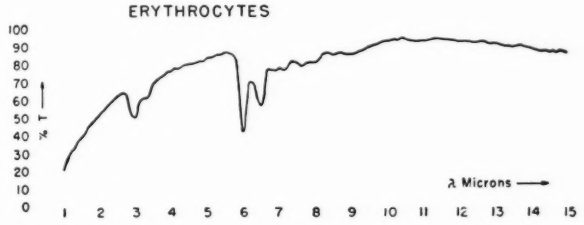

FIG. 15.—Erythrocytes (human)

FIG. 16.—Fat (human)

FIG. 17.—DBA mouse liver

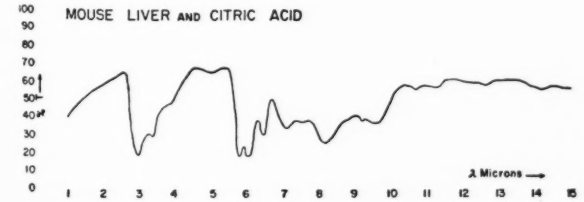

FIG. 18.—DBA mouse liver and citric acid mixture

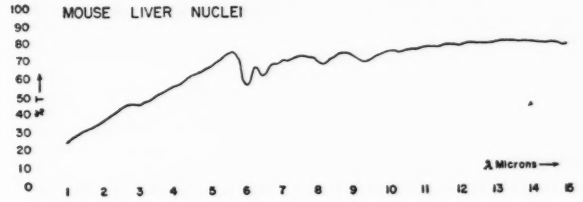

FIG. 19.—DBA mouse liver nuclei separated by the citric acid method and washed with isotonic NaCl.

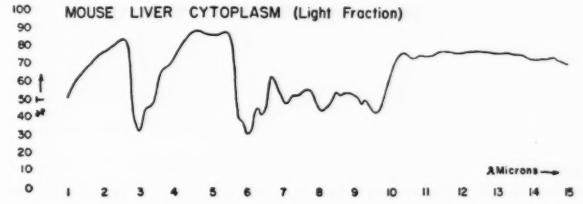

FIG. 20.—DBA mouse liver cytoplasm and citric acid. Light fraction.

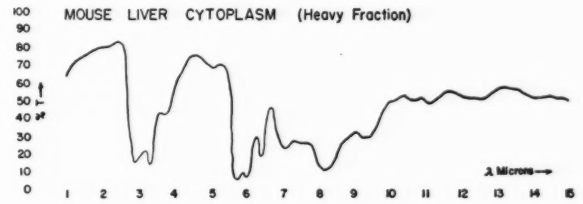

FIG. 21.—DBA mouse liver cytoplasm and citric acid. Heavy fraction.

FIG. 22.—Citric acid, *W*

FIG. 23.—Mouse tumor, mashed

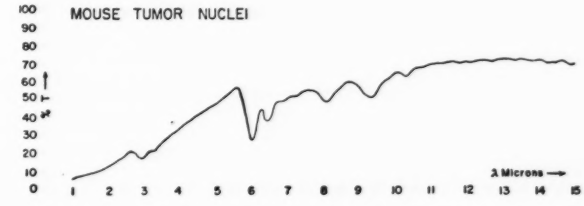

FIG. 24.—Mouse tumor nuclei separated by the citric acid method and washed with isotonic NaCl.

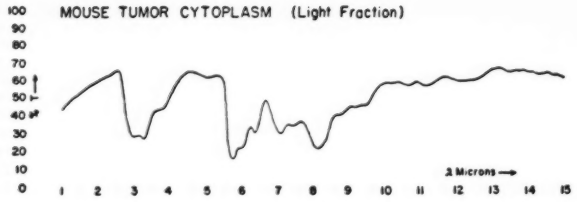

FIG. 25.—Mouse tumor cytoplasm and citric acid. Light fraction.

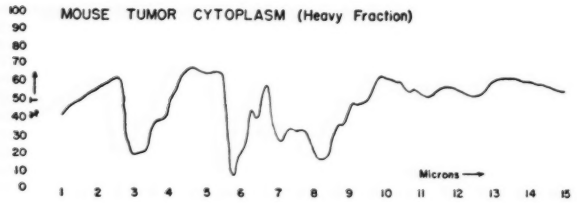

FIG. 26.—Mouse tumor cytoplasm and citric acid. Heavy fraction.

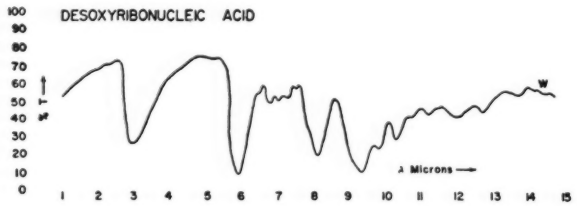

FIG. 27.—Desoxyribonucleic acid, film

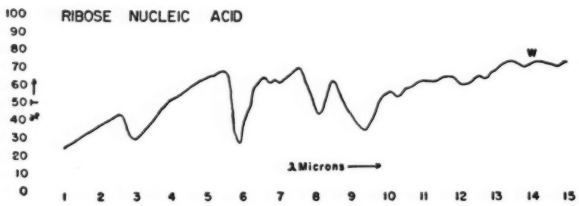

FIG. 28.—Ribonucleic acid, film

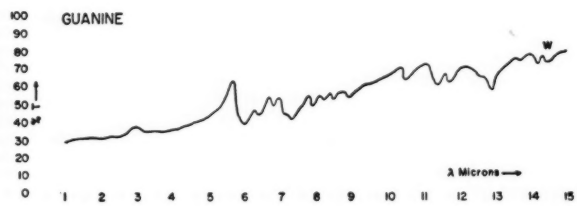

FIG. 29.—Guanine

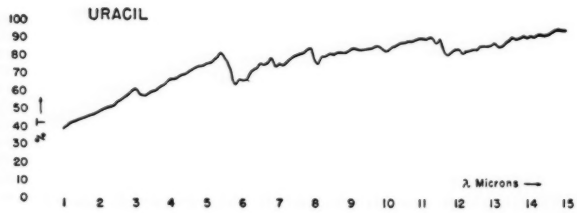

FIG. 30.—Uracil, *A*

FIG. 31.—Thymine

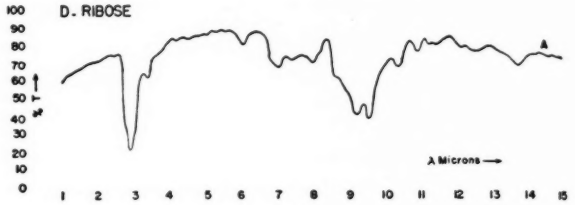

FIG. 32.—D-ribose

FIG. 33.—Cytidine

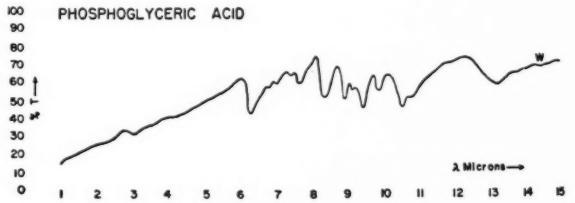

FIG. 34.—Phosphoglyceric acid

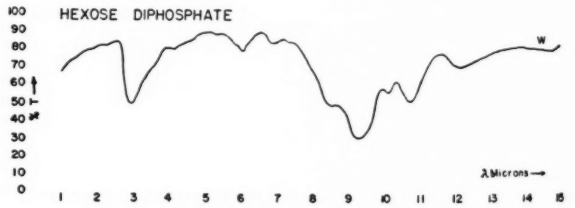

FIG. 35.—Hexose diphosphate

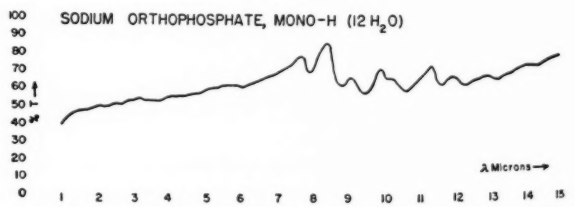

FIG. 36.—Sodium orthophosphate, $Na_2HPO_4 \cdot 12H_2O$, *W*

that of the lighter fraction. It has a broad band at 9.35 and a weak band at 10.15 μ. Mouse liver nuclei absorb at 8.15, 9.4, and 10.35 μ.

The mouse tumor has pronounced bands at 8.05 and 9.1, and weaker bands at 9.4 and 10.25 μ. The lighter fraction of tumor cytoplasm, which has bands at 9.2 and 9.45 μ, differs but slightly from that of the heavy fraction. Although the bands for mouse tumor nuclei in the interval 8–10.5 μ are more pronounced than for the mouse liver nuclei, the patterns are nearly identical.

A correlation with infrared absorption data of the percentages of constituents found in tissues by chemical methods is valuable in making absorption band assignments. For this reason chemical findings will be briefly discussed.

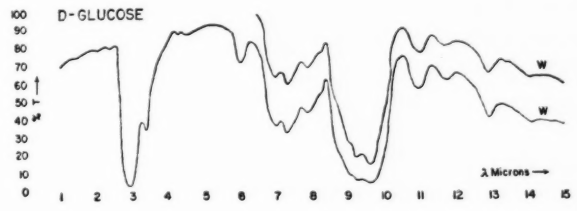

FIG. 37.—D-glucose curves for two different sample thicknesses.

A living cell contains 70–80 per cent water. The remainder of the cell is chiefly protein in one form or another. On the average, cell nuclei in bulk contain about 30 per cent desoxyribonucleic acid (DNA) and 10 per cent lipid on a dry-weight basis (4). Data available at the present time seem to bear out the fact that DNA is confined exclusively to the nucleus. Other constituents of the nucleus are a basic protein such as protamine or histone, proteins of higher order, ribonucleic acid (RNA), phosphorus compounds, and inorganic elements. The concentration of RNA in the nucleus varies between approximate limits of 3 and 30 per cent of the DNA concentration. A rough average figure for the RNA would be 10 per cent of the DNA concentration. Thus, on the average, cell nuclei would contain about 3 per cent RNA on a dry-weight basis.

In general, the average density of nucleic acids is greater in the nucleus than in the cytoplasm. However, since the nucleus generally has a smaller volume than the cytoplasm, the total amount of nucleic acids in the cytoplasm can be greater than that in the nucleus. In rat liver cells the RNA in the cytoplasm has been estimated from Schneider's data (17) to about 2.5 times the amount of DNA in the nucleus.

The RNA/DNA ratio (3, 15) for liver and pancreas is greater than 1; for brain, kidney, skeletal, and cardiac muscle, approximately 1; and for thymus and lung tissue it is less than 1, i.e., about 0.5. There is considerable variation in the total nucleic acid content of tissues on the basis of biochemical data. Thymus, spleen, and pancreas are rich in nucleic acid, while brain and fibrous tissue such as muscle tissue are low.

Marshak (13) determined the volume of cells and nuclei of mouse liver to be about 12.1×10^{-9} cm.^{3} and 7.3×10^{-10} cm.^{3}, respectively. These values yield a nuclear-cytoplasmic ratio of 0.064. This means, on the average, in the case of a living liver cell with a weight of RNA in the cytoplasm equal to 2.5 times the weight of DNA in the nucleus, that the RNA is distributed in a volume of cytoplasm about 15 times the volume of the nucleus. The concentration of the RNA in the cytoplasm in a nondehydrated cell would then equal $\frac{1}{6}$ that of the DNA in the nucleus. On the basis that 30 per cent of the nucleus (dry weight) is DNA, the RNA is estimated to be 5 per cent of the dry weight of the cytoplasm. In this step it is

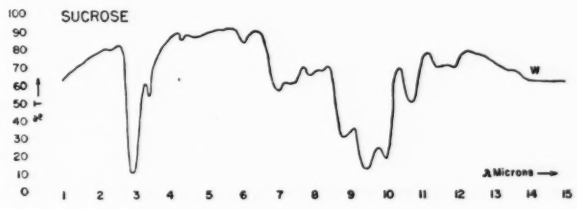

FIG. 38.—Sucrose, film

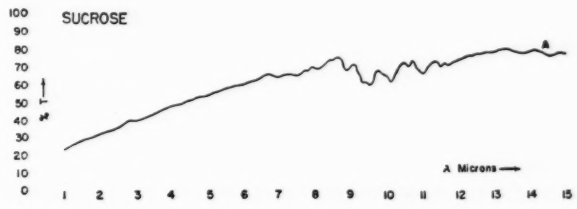

FIG. 39.—Sucrose, powder

implicitly assumed that the density of the cytoplasm is equal to that of the nucleus.

Rat liver cells contain approximately 10 per cent nuclear and 90 per cent cytoplasmic material on a dry-weight basis (16). If the nucleus is 30 per cent DNA (dry weight) and if the amount of RNA in the cytoplasm is equal to 2.5 times the weight of DNA in the nucleus, then the RNA is estimated to be about 8.3 per cent of the dry weight of the cytoplasm. This figure is in rough agreement with the value calculated from the nuclear-cytoplasmic ratio.

The purpose of these crude calculations is to obtain an idea of the order of magnitude of the concentrations of nucleic acid in dried cytoplasm.

The results of chemical determinations are usually presented in terms of RNA-phosphorus/100 mg tissue and do not explicitly state the required information. This method of reporting is preferred, since the proportions of the nucleotides in nucleic acids of different origin may vary (15).

There are many types of tissue rich in nucleic acids which have a higher value than liver for the nuclear-cytoplasmic ratio (7). One example is dbrB tumor. Here the ratio is roughly 1:2.

DNA and RNA have strong absorption bands at 8.1 and 9.35 μ, along with a fairly pronounced band at 10.3 μ. It is interesting to note that DNA has a band at 9.8 μ which distinguishes it from RNA.

From the facts related in the preceding discussion of nucleic acids, it is evident that these substances are often present in sufficient quantity to cause appreciable absorptions at 8.1, 9.35, and 10.3 μ. The striking resemblance of the absorption patterns of DNA and RNA, mouse liver and mouse tumor nuclei, and of many tissues both neoplastic and normal in the vicinity of the above three bands is strong evidence that an appreciable amount of this absorption is due to nucleic acids. This substantiates a statement by E. Blout and R. Mellors (2), to the effect that an absorption band at 9.3 μ "lies at the same position as one of the strong ones in the nucleic acid spectra, and it is probable that this increase in absorption is due to an increased amount of nucleic acids in the rapidly proliferating and more cellular carcinoma." Furthermore, the absorption intensities at these wave-lengths for many types of tissue are correlatable with the nucleic acid concentrations in the cell. It is seen from the curves, for example, that the muscle tissue sample does not present the pronounced absorption pattern in the 8–11-μ interval characteristic of the liver metastasis.

The question arises concerning what constituents of the nucleic acids are responsible for the nucleic acid pattern in the region 8–11 μ, i.e., the absorption bands at 8.1, 9.35, and 10.3 μ. From a survey of Charts 27–39, as well as of complementary curves available in the literature and otherwise accessible (1),[1] reasonable conclusions can be drawn regarding these constituents.

Purine and pyrimidine bases, such as adenine, guanine, uracil, and thymine, do not appear to have absorptions of sufficient intensity at the right wave-lengths to produce the above nucleic acid pattern. However, the pentose sugars, ribose and desoxyribose, and nucleosides, such as cytidine, display sufficiently strong bands in this region to account for an appreciable amount of the absorption. The latter is true for many phosphoric compounds. From these data it is inferred that the nucleic acid pattern is mainly due to the pentose and phosphoric acid constituents.

Such a statement has less meaning for vibrations in the skeletal region than for those at lower wave-lengths. Nevertheless, plausible reasons for such an assertion are that coupling between the absorbing vibrations in the purine or pyrimidine bases and the absorbing vibrations of the pentose and phosphoric acid constituents may be small and that the amplitudes of the latter vibrations appear to be overwhelming when compared to the amplitudes of the former in the region 8–11 μ. It also appears that the absorbing vibrations of the pentose and phosphoric acid components in this interval are comparable in amplitude and to a large degree simultaneous and that the act of differentiating between the two in combination cannot be performed.

Since other carbohydrates (10), e.g., glucose, exhibit intense absorptions somewhat similar to those of pentose sugars, members of this class of compounds could be the source of interference if present in concentrations comparable to those of nucleic acid. Infrared data for amino acids show that these constituents of proteins do not have patterns similar to those of nucleic acid in the region 8–11 μ.

While the ultraviolet bands of nucleoproteins at about 2,600 A are attributed to conjugated double-bond systems of purine and pyrimidine rings, it is much more plausible to attribute the characteristic infrared absorption of these compounds in the interval 8–11 μ to the pentose and phosphoric acid constituents. Thus, the two technics should yield complementary as well as substantiating information in this regard.

SUMMARY AND CONCLUSIONS

Infrared absorption curves in the region 1–15 μ for typical normal tissues, neoplastic tissues, and important constituents of tissues have been determined. These data have been analyzed to ascertain the relationship between the absorption bands and sample composition.

The following conclusions have been drawn:

1. The infrared absorption pattern of ribo- and desoxyribonucleic acids consisting of the 8.1, 9.35, and 10.3 μ bands is due mainly to the pentose and phosphoric acid components.

2. RNA and DNA are present in many tissues in sufficient concentrations to cause appreciable absorption in the region 8–11 μ.

3. The absorption pattern of mouse liver and

[1] D. L. Woernley, Infrared Absorption Curves, unpublished.

tumor nuclei, and of many tissues both neoplastic and normal, strongly resembles that for DNA and RNA in the region 8–11 μ.

4. Absorption intensities at approximately 8.1, 9.3, and 10.3 μ can be correlated with the concentrations of nucleic acids in tissues.

5. Highly cellular neoplastic and normal tissues rich in nucleic acids produce strong and characteristic absorption in the 8–11-μ region, while muscular tissue generally displays less pronounced absorption bands. Mouse brain appears to be an exception, but further study may account for the discrepancy.

6. The absorption pattern for tissues in the 8–11-μ region is subject to many sources of interference. Carbohydrates, such as gluclose, and many phosphoric compounds have intense bands in this region. Fats and proteins such as occur in whole blood, blood serum, and erythrocytes absorb in this region, but the absorptions are not very pronounced.

ACKNOWLEDGMENTS

The major portion of the technical work in this investigation was carried out by Miss B. J. Kress. R. F. Pfeiffer and Miss M. J. Powers also worked on certain phases of the problem.

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Enzymatic Deamination of 8-Azaguanine in Normal and Neoplastic Tissues*†

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In 1949, Kidder and collaborators (9) demonstrated that 8-azaguanine strongly inhibited the growth of several transplanted mouse tumors. Following this report, the effect of 8-azaguanine on the growth of many experimental neoplasms has been studied in this and other laboratories (1, 4, 6, 10, 12, 17–19). With one exception, the August carcinoma, all rat tumors were resistant to the carcinostatic action of this compound. On the other hand, the only rabbit tumor which has been studied, the Brown-Pearce carcinoma, was significantly inhibited. In the mouse, the species of choice for most of the investigations, this chemotherapeutic agent was effective against a variety of carcinomas and certain leukemias but not against other leukemias, melanomas, and sarcomas.[1]

The present investigation was undertaken in the hope of finding a reason for this striking dichotomy between azaguanine-susceptible and azaguanine-resistant tumors. It had been established in preliminary experiments that an enzyme system present in mouse and rabbit tissues catalyzed the deamination of 8-azaguanine to 8-azaxanthine (5,7-dihydroxy-1*H*-*v*-triazolo [*d*] pyrimidine) (5); proof of the identity of the end-product of this deamination as 8-azaxanthine, by filter paper chromatography and comparative ultraviolet spectroscopy, has been recently described (11).

* This investigation was supported (in part) by a research grant (C-1386) from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

† A preliminary report on this investigation was given at the forty-second annual meeting of the American Association for Cancer Research, Inc., Cleveland, Ohio, April, 1951 (8).

‡ Postdoctoral research fellow, National Cancer Institute, National Institutes of Health, Public Health Service, 1950–1951.

§ Present address: Basic Research Laboratory, Veterans Administration Hospital, Brooklyn, N.Y.

[1] One group of investigators (4) has reported a positive effect of 8-azaguanine against mouse Sarcoma 37, but the dosage levels were so high and the differential between carcinostasis and systemic toxicity was so slight that an interpretation of these results has not been attempted.

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More extensive data on the deamination of 8-azaguanine by a guanase preparation were reported by Roush and Norris (15). The data to be presented suggest that the activity of this enzyme is an important factor in determining the response of various tumors to 8-azaguanine.

MATERIALS AND METHODS

Experimental material.—The following transplantable tumors were employed: Sarcoma 180 in Paris RIII or C57 mice; mammary adenocarcinomas 755 and E 0771 in C57 mice; mammary adenocarcinoma RC in DBA mice; lymphosarcoma 6C3HED in C3H mice; myelogenous leukemia C1498 in C57 mice; Brown-Pearce carcinoma in rabbits. The details of tumor transplantation, response to 8-azaguanine, and tumor growth measurements have been reported previously (6). For the present experiments, tumors were allowed to grow without treatment until they reached a suitable weight without evidence of necrosis; only healthy, non-necrotic tissue was used. Each sample represented a pool of tissue from two to five rabbits or ten to twenty mice.

Normal tissues were obtained from both normal and tumor-bearing mice and rabbits. The livers were routinely pooled after removal of the gallbladder and perfusion with or washing in cold saline. Animals were killed with chloroform or nembutal; it was ascertained in preliminary experiments with other methods of killing, i.e., air injection or decapitation, that anesthetic agents had no effect on the azaguanine deaminase activity of normal or tumor tissue.

Almost without exception, analyses were carried out immediately after removal of the tissue from the animals; however, rapid freezing of the tissues in a deep-freeze, followed by slow thawing at room temperature at a later time, did not appear to cause any decrease in enzyme activity.

Preparation of homogenates.—The pooled tissue was cut into small pieces, and a weighed sample was homogenized for 15 minutes in a Potter all-glass homogenizer with hypertonic (0.77 M) sucrose in 0.01 M phosphate buffer at pH 7.2. The final concentration of tissue in the homogenate was routinely 15 per cent on a wet weight basis. All operations were carried out at a temperature ranging from 0 to 4° C.

The homogenate was subdivided into aliquots for nuclear count, determination of azaguanine deaminase activity, and other analyses of interest.

Determination of azaguanine deaminase activity.—Enzyme activity was assessed by determination of initial linear reaction rates on the basis of a microdetermination of the ammonia evolved (16) when the homogenate was incubated with substrate for varying periods of time. The procedure is, in brief, as follows: 1 ml. of homogenate (= 150 mg. fresh tissue) was incubated with 1 ml. of 8-azaguanine solution (= 5 mg. 8-

azaguanine[2]) in a small side-arm test tube maintained at 37° C. in a water bath. A fresh substrate solution was prepared each time by suspending the required amount of 8-azaguanine in half the required volume of distilled water and dissolving it by the addition of one or two drops of 50 per cent NaOH; the resulting solution was adjusted to pH 8.0 with 2 N HCl and diluted to mark with water.

The enzyme reaction was stopped by the addition of 3 drops of 8 N H_2SO_4. Each incubation tube was then connected by means of rubber tubing to another side-arm test tube containing 2 ml. of 2 per cent H_3BO_3 + 0.02 ml. of indicator (8 parts 0.1 per cent bromcresol green and 1 part 0.1 per cent methyl red in 95 per cent ethanol). The incubation mixture was made alkaline with 1 ml. of half-saturated K_2CO_3, and the ammonia liberated was aspirated for 20 minutes into the boric acid solution. The latter was back-titrated to its original pink color with standard dilute H_2SO_4 in a 1-ml. total capacity ultramicroburette (E. Greiner & Co.) of modified design.

Control tubes in which 1 ml. H_2O was substituted for the substrate solution were incubated for the same length of time and provided a measure of the blank caused by nonspecific ammonia production in the homogenates. All values were corrected for the corresponding blanks and expressed as μg ammonia nitrogen/hour/ml homogenate. From these values, deaminase rates were calculated on a cell basis (μg ammonia nitrogen/hour/10^6 cells).

Calculation of results on a cell basis.—Almost all the results of studies on the biochemical composition of various tissues in the past have been expressed on the basis of nitrogen or units of fresh or dry weight of tissue. Recently, Davidson and Leslie (2, 3) and Price and Laird (13) have demonstrated the advantages of using the number of cells in a sample as the basic unit of calculation in analyses of intracellular components. This concept has been applied here; an evaluation of this approach and a report on the requisite methods and on results obtained on a variety of normal and neoplastic tissues will be presented elsewhere.[3]

The number of cells in a sample of tissue prepared for determination of azaguanine deaminase activity was ascertained by direct enumeration of nuclei in an aliquot of the same homogenate. For this purpose, duplicate 1-ml. aliquots were diluted (1:60) with 3 per cent acetic acid containing 0.02 per cent methyl green, and the nuclei in each final suspension were counted in duplicate in a hemacytometer (14). It was assumed that each nucleus represented one cell. It is, of course, recognized that the results obtained by this general procedure represent average values for the various kinds of cells which are present in each tissue.

RESULTS

Properties of the enzyme preparations.—It has been pointed out that the determination of azaguanine deaminase in tissue homogenates is based on the initial linear reaction rate. Chart 1 demonstrates that the rate of evolution of ammonia nitrogen in homogenates of different activity is a linear function of time and illustrates the type of experimental data upon which the present report is based. Homogenates of tissue with a high deaminase activity were routinely incubated for 30, 60, and 90 minutes, whereas the incubation period was prolonged to 180 minutes for homogenates of low activity.

Experiments on the effect of dilution of homogenate on the rate of enzymatic deamination of 8-azaguanine demonstrated that this rate was proportional to the amount of tissue present in the homogenate.

Preliminary experiments on the localization of azaguanine deaminase within the cell indicate that the enzyme is not associated with the nuclear fraction.

Lack of carcinostatic activity of 8-azaxanthine.—The effect of 8-azaxanthine on a variety of transplanted tumors was investigated with the same methods described in the experiments with 8-azaguanine (6). The appreciably greater solubility of the dihydroxy derivative and its lack of toxicity to mice at daily doses as high as 500 mg/kg permitted the administration of 4–6 times as great an amount of 8-azaxanthine as of 8-azaguanine. Nevertheless, no inhibition of growth of any of the tumors under investigation was observed (Table 1).

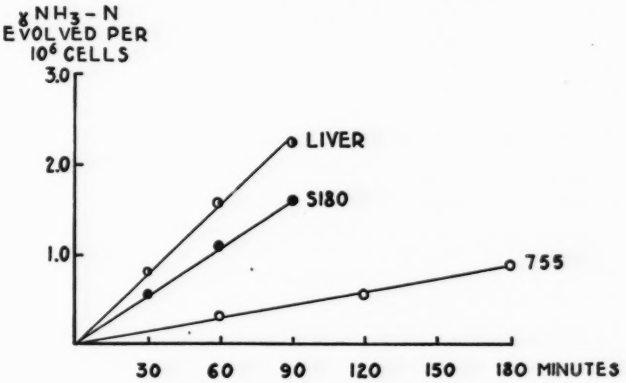

CHART 1.—Rate of evolution of ammonia nitrogen in homogenates of different tissues as a function of time.

In the enzymatic deamination of 8-azaguanine, therefore, a compound which was strikingly carcinostatic for four of the seven tumors under investigation was converted to a derivative which failed to exhibit any inhibitory activity against any of the seven tumors.

Azaguanine deaminase activity of normal tissues.—Table 2 summarizes the data obtained on various tissues of normal and tumor-bearing mice and rabbits. In mice, the values ranged from the low deaminase activity of kidney to the extremely high activity in intestine;[4] among the rabbit tissues

[2] Lederle 8-azaguanine, batch no. 7-7242, was used in these experiments; ultraviolet spectroscopic analysis indicated a purity of 85 per cent. In preliminary experiments, a pure sample of 8-azaguanine obtained through the courtesy of Dr. Morris Engelman was used for comparative purposes.

[3] Manuscript in preparation.

[4] For these determinations, the small intestine was used after removal of the intestinal contents. A separate experiment on the latter indicated that the small amounts of intestinal contents which might still have been present in the homogenate might have been responsible for less than 5 per cent of the total deaminase activity of this tissue.

investigated, only liver and intestinal mucosa exhibited measurable activity.

Experiments with blood were carried out in both species. The blood was allowed to clot; the clot was briefly homogenized and subjected to freezing and thawing. In a typical experiment, 1 ml. of mouse blood caused the production of more

TABLE 1

EFFECT OF 8-AZAXANTHINE ON A VARIETY OF TRANSPLANTED TUMORS*

Tumor	Daily dose of 8-azaxanthine (mg/kg)	Mean tumor weight or length of survival time: Control group	Azaxanthine-treated group	Azaguanine-treated group
755	50	394 mg.	450 mg.	
	300	729 mg.	652 mg.	34 mg.
RC	300	741 mg.	709 mg.	494 mg.
E 0771	300	700 mg.	625 mg.	278 mg.
Brown-Pearce	100	547 mg.	456 mg.	178 mg.
Sarcoma 180	50	617 mg.	506 mg.	
	300	493 mg.	528 mg.	490 mg.
6C3HED	50	17.7 days	17.1 days	15.4 days
C1498	300	12.0 days	11.3 days	11.7 days

* A preliminary report on some of these data has been presented (5). The daily dose of 8-azaguanine was 50 mg/kg for mice and 25 mg/kg for rabbits; the response of all the tumors to this agent was the expected one.

TABLE 2

AZAGUANINE DEAMINASE ACTIVITY OF NORMAL TISSUES

Species	Tissue	No. of experiments	Deaminase rate: Av.	Range
			μg NH_3-N/hour/10^6 cells	
Normal mice	Liver	4	2.42	1.00–2.92
	Kidney	3	0.56	0.33–0.70
	Brain	3	4.18	1.81–5.37
Tumor-bearing mice*	Liver	17	2.48	1.35–4.88
	Intestine	2	>16	
Normal rabbits	Liver	4	3.06	2.20–4.34
	Kidney	2	0†	
	Intestinal mucosa	1	0.53	
	Testis	1	0	
Tumor-bearing rabbits	Liver	5	1.84	0.61–3.38
	Testis	1	0	
	Spleen	1	0	

* All groups of mice, regardless of the identity of the tumor present, are included.

† No measurable deamination in 180 minutes.

than 400 μg. ammonia nitrogen in the first hour (100 per cent deamination = 620 μg. ammonia nitrogen). The enzymatic activity of the corresponding serum was very low; this finding indicated that azaguanine deaminase was associated with the erythrocytes. There was no measurable activity in rabbit blood or serum.

In view of the significant deaminase activity of mouse blood, it was of interest to determine whether the amount of blood remaining in the tumors upon their removal from the animals represented an appreciable source of *in vitro* deaminase in these homogenate experiments. A pooled sample of 755 tumor, the mouse neoplasm with the lowest deaminase activity, was homogenized in distilled water, and the hemoglobin content of the supernatant fluid after prolonged high-speed centrifugation (32,000 × *g*) was determined. Suitable calculations from the hemoglobin content of this homogenate led to the conclusion that the amount of blood remaining in the tumor after the death of the host could not have been responsible for more than 10 per cent of the deaminase activity of this tumor. The hemoglobin content of a similar homogenate of Sarcoma 180 was negligible.

Azaguanine deaminase activity of various tumors.—Chart 2 shows the results obtained with azaguanine-susceptible and azaguanine-resistant

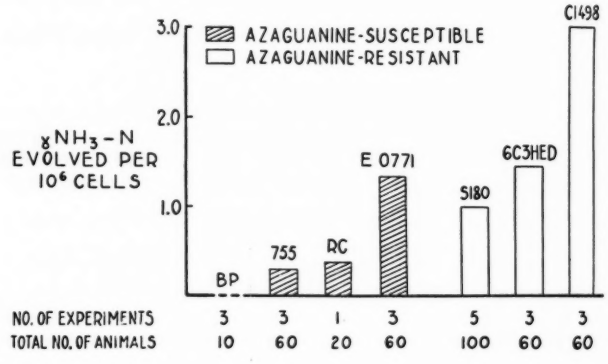

CHART 2.—Azaguanine deaminase activity of various transplanted tumors. The value for the Brown-Pearce tumor (BP) is zero. All values are calculated on the basis of a 1-hour incubation.

tumors. The following observations may be made: (*a*) the Brown-Pearce tumor appears to be qualitatively different from the other tumors, since it does not have measurable azaguanine deaminase activity, even when the time of incubation was extended to 5 hours; (*b*) in homogenates of the three tumors resistant to 8-azaguanine the *in vitro* deamination of the carcinostatic agent to an inactive derivative proceeds at a rapid rate; (*c*) homogenates of three of four tumors susceptible to the drug exhibit a low or negligible deaminase activity. It may, then, be postulated that azaguanine deaminase is an important factor in the cellular control of the level of the carcinostatic agent and that it plays a part in determining the response of a particular tumor to the drug.

This hypothesis does not seem to hold for the E 0771 tumor, which exhibits both a high azaguanine deaminase activity and a pronounced susceptibility to the drug. At the present time we have no evidence to explain this discrepancy.

In the experiments summarized in this report, each tumor was obtained from a different group of mice. It was of interest to ascertain whether the presence of an azaguanine-resistant tumor in the same hosts with an azaguanine-susceptible tumor exerted any effect on the deaminase activity of the latter and vice versa. In one experiment, Sarcoma 180 and the 755 tumor were implanted into the same mice, the resistant tumor into one axilla and the susceptible tumor into the other. The azaguanine deaminase activities of Sarcoma 180 and 755 tumor in this experiment (cf. Chart 1) were 0.94 and 0.30 μg ammonia nitrogen/hour/10^6 cells, respectively (average of all samples of Sarcoma 180 and 755 tumor: 0.98 and 0.27 μg ammonia nitrogen/hour/10^6 cells, respectively). The presence of a second tumor in the same hosts, then, had no effect on the deaminase activity of either tumor.

DISCUSSION

The results of this investigation, which was based in its entirety on the measurement of *in vitro* deamination of 8-azaguanine by tissue homogenates, lead to certain inferences concerning the *in vivo* action and metabolism of this chemotherapeutic agent. The data which have been reported are consistent with the following conclusions: (*a*) a major portion of any therapeutic dose of azaguanine following injection into the tumor-bearing animals is rapidly deaminated by liver, brain, and other organs and thus rendered inactive as a carcinostatic agent; (*b*) an increasingly minute portion of the drug reaches the cells of the tumor as this "detoxification" in the normal tissues continues; (*c*) the cells of some of the tumors have a sufficiently high azaguanine deaminase activity so that the intracellular level of the drug is kept below the threshold concentration needed for inhibition of tumor growth, whereas the deaminase activity of the cells of other tumors is too low to afford them this protection. The clear-cut dichotomy in the susceptibility of various tumors to 8-azaguanine could be explained on this basis.

In a concomitant study in this laboratory (7),[5] it has been demonstrated by means of filter paper chromatography and quantitative ultraviolet spectroscopy that 8-azaguanine is very rapidly deaminated *in vivo* to 8-azaxanthine and that both compounds are rapidly excreted. The metabolic significance of the high rates of *in vitro* deamination of 8-azaguanine by homogenates of normal tissues has thus been confirmed. It cannot yet be decided whether the observed difference in azaguanine deaminase activities between azaguanine-susceptible and azaguanine-resistant tumors constitutes the only factor which determines the response of a tumor to the drug or whether it provides only a partial answer. In view of the findings with mammary carcinoma E 0771, the second alternative is the more likely one. The available evidence indicates, however, that enzymatic control of cellular levels of 8-azaguanine is an important factor in the success or failure of tumor chemotherapy with this drug.

[5] Full manuscript in preparation.

SUMMARY

1. The enzymatic deamination of 8-azaguanine by homogenates of normal and neoplastic tissues of mice and rabbits has been studied.

2. This reaction transforms 8-azaguanine, which inhibits the growth of four of the seven tumors under investigation, into 8-azaxanthine, which has no carcinostatic activity for any of the seven tumors.

3. The distribution of azaguanine deaminase in normal tissues has been reported; the activity of liver and other tissues is sufficiently high to cause the rapid inactivation of major portions of injected 8-azaguanine.

4. The azaguanine deaminase activity of the three azaguanine-resistant tumors is high; that of three out of four azaguanine-susceptible tumors is low or negligible.

5. The significance of these findings for an assessment of the different response of different tumors to 8-azaguanine has been discussed.

ACKNOWLEDGMENTS

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Metabolism of Pyruvate in Tumor Homogenates*

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In 1948, LePage (3) noted that, in tumor homogenates undergoing active anaerobic glycolysis, considerable pyruvate disappeared from the pyruvate-lactate pool. This problem was investigated further by Stoesz *et al*. (10), who made the first attempts at determining the products of the reaction. Under optimal conditions, the "pyruvate disappearance" approximated one-third of the lactate production, required diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP), and was of the same magnitude both aerobically and anaerobically. Using C^{14}-labeled pyruvate, Stoesz *et al.* (10) also found that only 5 per cent of the "pyruvate disappearance" could be accounted for by decarboxylation reactions and that no pyruvate was converted to oxaloacetate.

The purpose of these studies has been to determine the best conditions under which the reactions concerned with "pyruvate disappearance" occur *in vitro* and to establish the product or products resulting therefrom. The final optimal chemical medium used was found to differ only slightly from that described for maintaining phosphate bond energy in tumor homogenates (3).

METHODS AND RESULTS

For these *in vitro* studies, multiple transplants of the Flexner-Jobling carcinoma in female rats of the Holtzman-Rolfsmeyer strain were used throughout. The tumors were used 9–11 days after transplantation, since it was found that maximum activity was obtained when the tumors were essentially free of necrotic material and actively growing. The tumor weights ranged from 750 to 1,250 mg.

Immediately before each experiment, the tumor-bearing rat was decapitated, and the tumors were rapidly excised and placed in cold, isotonic KCl. After they had been freed of connective tissue and weighed, the tumors were homogenized in 9 volumes of cold, isotonic KCl in Potter-Elvehjem homogenizers.

The reactions were carried out in Warburg respirometer vessels, to which all the necessary constituents had been added previous to 0.3 ml. of the homogenate. The final volume in each reaction vessel was 3.0 ml. The flasks were incubated at 38° C. for 60 minutes, and the reactions were stopped by the addition of 0.25 ml. of 26 per cent perchloric acid from the side-arm. All experiments were carried out under anaerobic conditions achieved by the evacuation technic (11).

It is desirable to work as rapidly as possible after the animal is killed, since the system under study is relatively labile, as illustrated by data in Table 1, which compares the "pyruvate disappearance" system to the glycolytic system.

Highly purified preparations of triphosphopyridine nucleotide (TPN) and concentrates of coenzyme A had no effect upon the fresh homogenates with regard to "pyruvate disappearance" and had no restorative effect upon the incubated homogenates.

TABLE 1

THE STABILITY OF THE "PYRUVATE DISAPPEARANCE" SYSTEM AS COMPARED TO THE GLYCOLYTIC SYSTEM

The homogenate was incubated at 38° C., then used in a routine 60-minute experiment. Each figure is an average calculated from duplicate flasks from each of two comparable experiments.

Incubation time (min.)	Lactate production	Net P uptake (μM per flask)	Pyruvate disappearance
0	8.0	5.1	5.8
5	8.0	4.3	4.5
15	6.1	2.6	2.5

The evaluation of the components in the medium is shown in Charts 1–8 and was based upon the measurements of CO_2 evolution, phosphate uptake, and lactic acid production (cf. LePage and Umbreit [11]). Measurement of the extent of "pyruvate disappearance" was based on analyses for pyruvate uptake (2) and lactate production in the glycolytic system, blocked by fluoride at the phosphoglyceric acid step to the extent of 85–90 per cent (described below). Because of the fluoride block, pyruvate was added to the medium to act as hydrogen acceptor.

The medium of LePage (3) was used at first, and the components varied one at a time to determine optima. Charts 1–8 are plotted so that the amount of "pyruvate disappearance" from the pyruvate-lactate pool is related to the amount of each component *added* to each flask.

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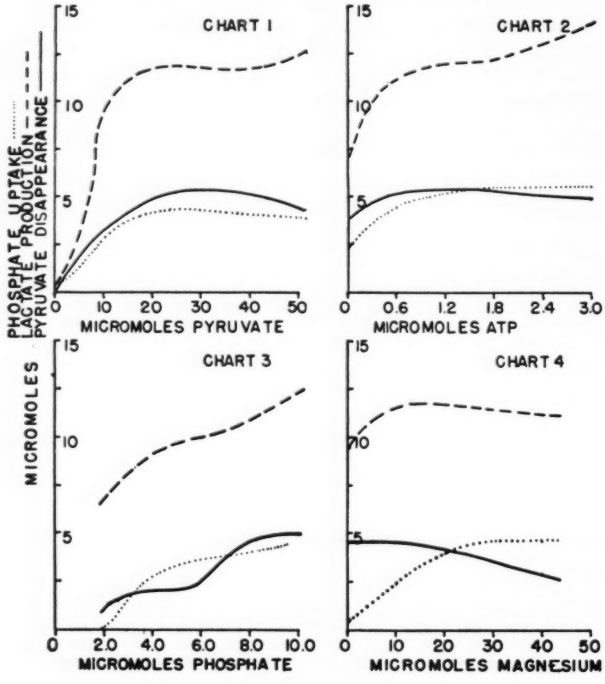
Pyruvate.—With fluoride present, added pyruvate serves as a hydrogen acceptor. Chart 1 shows that lowering the level of pyruvate tends to slow down the entire system, and higher than optimal levels decrease the amount of pyruvate that disappears. This may be due to a slight toxicity of the added pyruvate.

ATP[1] *and Inorganic Phosphate.*—The need for phosphorylating conditions for "pyruvate disappearance" is seen in Charts 2 and 3. ATP is required to a much greater extent than is shown by the chart, since the presence of adenine nucleotide was demonstrated in the hexosediphosphate (HDP)[2] used in this experiment. When HDP, which had been purified by ion-exchange chromatography on Dowex-1 (Cl form), was used, however, the addition of 1 μM of ATP per flask then stimulated the "pyruvate disappearance" by 250 per cent.

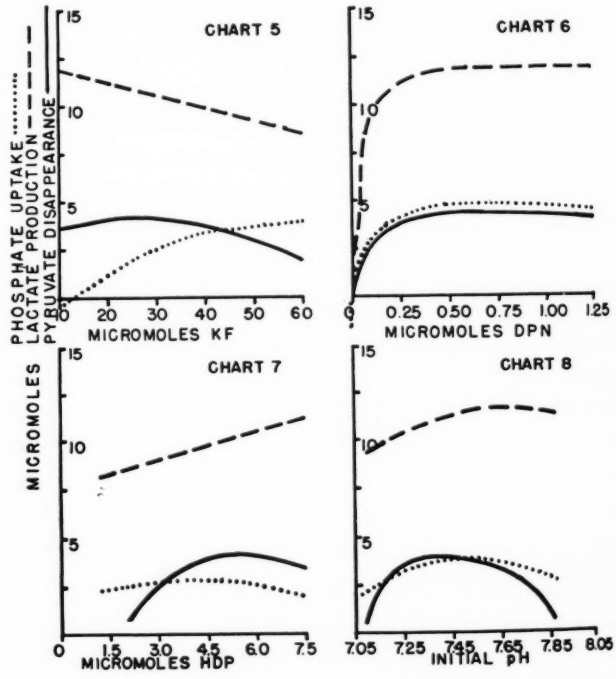


CHARTS 1–4.—The effects of varied concentrations of pyruvate, ATP, inorganic phosphate, and magnesium ion upon "pyruvate disappearance," lactate production, and phosphate uptake.

As the level of inorganic phosphate is increased, a point is reached where the amount of "pyruvate disappearance" increases at a rate greater than the increase in rate of lactate production.

Magnesium Ion.—Chart 4 shows the effect of added magnesium ion upon the system. If magnesium ion is required for "pyruvate disappearance" reactions to take place, it is needed only in minute concentrations, since its effect upon glycolysis is much more marked.

Fluoride.—As is shown in Chart 5, fluoride has a multiple purpose: to partially inhibit glycolysis, to increase phosphorylative efficiency due to inhibition of phosphatases, and to stimulate "pyruvate disappearance." At higher levels, however, the latter system is also inhibited. In addition, fluoride addition permits a quantitative estimation of the extent of the "pyruvate disappearance," since little pyruvate is contributed to the pyruvate-lactate pool from triosephosphate metabolism. Since the degree to which fluoride inhibited at the phosphoglyceric acid step was important in determining the amount of "pyruvate disappearance," experiments were carried out in which only priming levels of pyruvate were used, and the lactate production was measured at the various levels of fluoride. These data are presented in Table 2.



CHARTS 5–8.—The effects of varied concentrations of KF, DPN, HDP, and $KHCO_3$ upon "pyruvate disappearance," lactate production, and phosphorus uptake.

Diphosphopyridine nucleotide.[1]—The effect of varied DPN concentration is shown in Chart 6. The requirement of DPN for "pyruvate disappearance" is seen to parallel closely that of the glycolytic system. When the glucose and hexose phosphate substrates were omitted, the system showed a definite requirement for reduced DPN.[3]

[1] Pure ATP and 90 per cent DPN were prepared in this laboratory according to standard methods (11).

[2] Obtained from Nutritional Biochemicals Corp. as the barium salt.

[3] Prepared by the method of Lehninger, J. Biol. Chem., **190**:345–59, 1951.

The addition of approximately 10 μM of DPN·2H in a typical experiment yielded 4.7 μM of lactate and 2.6 μM of "pyruvate disappearance" products. Oxidized DPN had no effect under these circumstances. The addition of lactate had no effect upon this system, either in the presence of reduced DPN without substrate, or in the complete medium.

Hexosediphosphate.—The effect of varying the concentration of HDP is shown in Chart 7.

Initial pH.—The initial pH measurements were made as described by LePage (3). It may be seen in Chart 8 that the amount of pyruvate which disappears is dependent upon hydrogen ion concentration, there being an effective working range of 0.5–0.6 pH units.

TABLE 2

FLUORIDE INHIBITION DATA FOR A 60-MINUTE INCUBATION AT 38° C.

KF-final molarity	Per cent inhibition at the phosphoglyceric acid step
0.00	0
0.00167	75
0.00333	80
0.00667	83
0.0100	88*

* This figure was used to calculate the corrected pyruvate-lactate pool in subsequent experiments.

The final medium selected for further experiments is given in Table 3.

A number of experiments were done in an attempt to characterize and isolate the products of the system described above. It seemed likely that the "pyruvate disappearance" reactions involved a reduction, as demonstrated by the following type of experiment. With the use of the complete medium minus glucose, and the addition of 6 μM of HDP, the system was run until it failed. All the HDP was shown to be gone, and no triosephosphate remained. Yet only 7.5 μM of lactate appeared instead of a theoretical 12. Hence, the unknown products must have been formed by the addition of the extra 9 micro-equivalents of hydrogen to pyruvate or lactate, giving a molecule with at least two more hydrogens than pyruvate per 3 carbon atoms from the pyruvate-lactate pool.

Experiments to determine the extent of mixing through the fluoride block were conducted with pyruvate-1-C^{14} and pyruvate-2-C^{14}.[4] Reaction mixtures were incubated 0(T_0) and 60(T_{60}) minutes and were inactivated with perchloric acid. The pyruvic acid was isolated from each flask as the 2,4-dinitrophenylhydrazone by the method of LePage (5). The isolated derivatives were plated on paper and the specific activity (sp. act.) determined, with corrections for the self-absorption of the paper plate and analyses for the pyruvate derivative on the plate. In the T_0 mixture, the pyruvate-1-C^{14} had a sp. act. of 1,650 counts/min/μM. This was checked by isolation of the phenylhydrazone derivative, which had a specific activity of 1,650 counts/min/μM. When pyruvate-2-C^{14} was used, the T_0 mixture had a sp. act. of 1,200 counts/min/μM (1,190 counts/min/μM upon combustion and counting as $BaCO_3$). In the T_{60} mixture, the dinitrophenylhydrazones had sp. acts. of 1,380 counts/min/μM and 1,060 counts/min/μM for pyruvate-1-C^{14} and pyruvate-2-C^{14}, respectively, which gave the ratios:

$$\frac{T_{60}}{T_0}(\text{pyruvate-1-C}^{14}) = 0.835;$$

$$\frac{T_{60}}{T_0}(\text{pyruvate-2-C}^{14}) = 0.890.$$

From the T_{60} mixtures, lactic acid was also isolated as zinc lactate, which was purified, combusted, and counted as $BaCO_3$. The ratios of the sp. acts. were as follows (using the sp. act. of the pyruvate for the T_0):

$$\frac{T_{60}}{T_0}(\text{pyruvate-1-C}^{14}) = 0.885;$$

$$\frac{T_{60}}{T_0}(\text{pyruvate-2-C}^{14}) = 0.915.$$

Average of all four determinations = 0.881.

Thus, the leak through the fluoride block at 0.01 M KF was approximately 12 per cent, in agreement with that determined by a different method described earlier.

Moreover, mixing through the block does not

TABLE 3

OPTIMAL MEDIUM FOR "PYRUVATE DISAPPEARANCE" WITH FLEXNER-JOBLING TUMOR HOMOGENATES

Constituent	Micromoles added per flask	Final molarity
Inorganic phosphate	10.0	0.0033
Magnesium ion	15.0	0.0050
DPN	0.65	0.00022
KF	30.0	0.010
HDP	5.0	0.0017
ATP	1.0	0.00033
Pyruvate	30.0	0.010
$KHCO_3$ (pH 7.4)*	7.5	0.0025
Nicotinamide	12.0	0.0040
Glucose	30.0	0.010

* The addition of $KHCO_3$ maintains the pH in the optimal range throughout the incubation.

[4] Prepared by the method of Anker (1) and further purified by ion exchange chromatography to free it of nonvolatile and neutral components.

occur, as shown by the finding that a negligible amount of radioactivity occurred in the Ba-insoluble phosphate esters, which contain the phosphoglyceric acid of the reaction mixtures. For example, with pyruvate-1-C^{14} of sp. act. 1,650 counts/min/μM 9.0 μM of lactate were found per flask and 3.0 μM of "pyruvate disappearance" products in a 60-minute incubation. Hence, greater than 12 μM of phosphoglyceric acid should be formed in order to account for the hydrogens. However, only 80 counts/min were found in the Ba-insoluble fraction out of 4,950 counts/min entering the "pyruvate disappearance" reactions.

Among the possibilities considered as a product of the "pyruvate disappearance" reactions was alanine. In an experiment with pyruvate-1-C^{14} (1,200 counts/min/μM), a "pyruvate disappearance" of 3.7 μM/flask was observed. Decarboxylation with ninhydrin was carried out by standard

TABLE 4

ELIMINATION STUDIES IN THE DETERMINATION OF THE PRODUCTS OF THE "PYRUVATE DISAPPEARANCE" REACTIONS

Experiment	Counts/min/flask found	Total counts/min entering the pyruv. disapp.	Per cent of total counts/min accounted for
Alanine production	129	4,440	2.9
Formate production	288	4,870	5.9
Decarboxylation to C_2 and C_1	110	3,000	3.7

methods (7, 12). A 1.5-ml. aliquot of the flask contents was neutralized to pH 4.8–5.0 and diluted to 2.0 ml.; 200 mg. of KH_2PO_4 and 100 mg. of ninhydrin were added, and the mixture boiled gently in a closed system for 30 minutes. The CO_2 was collected in alkali, precipitated, and counted as $BaCO_3$. In a T_0 control, 63 counts/min/flask appeared in the CO_2, whereas flasks incubated 60 minutes gave 192 counts/min/flask. Additional compounds reacting with ninhydrin to yield CO_2 formed in the incubation thus accounted for 129 counts/min/flask. Alanine formation could account for only 2.9 per cent of the pyruvate which disappeared. These data are summarized in Table 4, together with the results from the two following experiments.

Experiments were carried out to determine the amount of formate production. With pyruvate-1-C^{14}, the T_{60} reaction mixture was found to contain 8.9 μM of lactate, 12 μM of residual pyruvate, and 3.2 μM of unknown products. To a 1.5-ml. aliquot was added 1.0 ml. of glacial acetic acid and 250 mg. of mercuric acetate. The mixture was boiled 1–2 minutes in a reflux system, and the CO_2 was collected and counted. A correction was made with the use of a control containing the same amounts of lactate and pyruvate as the T_{60} mixture. The maximum amount of the "pyruvate disappearance" which could be accounted for as formate production was 5.9 per cent.

Another possible avenue for the disappearance of pyruvate is decarboxylation to C_2 and C_1 fragments, which was tested with pyruvate-1-C^{14} of sp. act. 1,030 counts/min/μM. In a control with boiled homogenate 84 counts/min/flask appeared in the CO_2, which was absorbed after the incubation, by injecting KOH through a rubber nipple on the side-arm vent of the respirometer vessel. In the flasks with actively metabolizing tissue, 2.9 μM of pyruvate disappeared per flask, and 194 counts/min appeared in the CO_2. Thus, only 3.7 per cent of the pyruvate which disappeared was decarboxylated (Table 4).

Since pyruvate might have been converted into C_4 acids by CO_2-fixation in these experiments, homogenates of liver and tumor were compared as to their rates of fixation. Duplicate flasks were used in each case, with $C^{14}O_2$ added as $K_2C^{14}O_3$. The reaction mixtures were deproteinized with perchloric acid after a 60-minute incubation and aerated vigorously for 1 hour; solid CO_2 was added, and the aeration was repeated. The solutions were then combusted and counted for radioactivity. Since TPN is needed for one of the CO_2 fixation reactions known to be present in liver (8), 200 μg.

TABLE 5

CO_2-FIXATION IN 40-MINUTE EXPERIMENTS WITH 15 MG. OF RAT LIVER HOMOGENATE OR 30 MG. OF FLEXNER-JOBLING CARCINOMA HOMOGENATE

	Minus TPN		Plus TPN	
	Counts/min/flask	μM $C^{14}O_2$ fixed	Counts/min/flask	μM $C^{14}O_2$ fixed
Tumor	62	0.010	770	0.12
Liver	5,463	0.89	7,350	1.19

of TPN were added per flask to corresponding flasks of the experiment. The addition of TPN did not affect the "pyruvate disappearance." The results appear in Table 5. It is evident that the fixation reaction is not significant in the tumor homogenates, under conditions which permit good CO_2-fixation in liver homogenates.

The four previous experiments suggested a 3-carbon chain in the products of pyruvate metabolism. It was noted earlier that these products must be reduced compounds with the addition of at least two hydrogens per 3-carbon unit. Hence, the possibilities to be considered must be neutral compounds or their phosphorylated derivatives. The

following procedure was adopted: after a 60-minute incubation in the presence of pyruvate-2-C^{14}, the flask contents were neutralized to pH 8.2 with concentrated KOH, and 0.1 ml. of 1 M $BaCl_2$ was added. The precipitate was collected by centrifugation in the cold. Four volumes of cold 95 per cent EtOH were then added to the supernate, and the precipitate was collected and washed with cold EtOH. The supernate was evaporated *in vacuo* to its original volume to remove the alcohol, then passed successively through columns of Dowex-50 (H form) and Dowex-1 (Acetate form). These three fractions, the Ba-insoluble, the Ba-soluble–alcohol-insoluble, and neutral fractions, respectively, were combusted and counted as $BaCO_3$.

TABLE 6

DISTRIBUTION OF RADIOACTIVITY IN THE PHOSPHATE ESTER FRACTIONS OF TUMOR HOMOGENATE EXPERIMENTS WITH PYRUVATE-2-C^{14}

Incubated 60 minutes

Experiment	μM pyruv. disapp./flask	μM of C^{14} found/flask: Ba-insoluble	Ba-soluble–alc.-insol.
I	4.11	0.241	2.88
II	4.63	0.109	2.13

The results from two such experiments are given in Table 6. Since the amount of radioactivity found in the neutral fraction did not exceed that found in T_0 mixtures, fractionated the same way, it was concluded that no free neutral metabolites of pyruvate were formed in this system, and this fraction was omitted from further consideration.

The Ba-soluble–alcohol-insoluble fraction contained radioactivity representing more than 50 per cent of the pyruvate which had disappeared, and a procedure was devised to account for a large proportion of the counts in this fraction. When these salts were dissolved in 1 N HCl and heated for 3 hours at 100° C., then reprecipitated with barium and alcohol, 18.5 per cent and 42.5 per cent of the radioactivity was retained in experiments I and II, respectively. The barium salt of 1,2-propanediol-1-phosphate (PDP)[5] was then added as carrier, and the salts were dissolved in 1 N HCl and heated again for 3 hours at 100° C. The solution was cooled, made 0.5 N with respect to KOH, and again heated for ½ hour at 100° C. This procedure hydrolyzes all sugar phosphates and all other known phosphate esters except that of propanediol. The solution was cooled, neutralized to pH 8.2, and basic lead acetate added in excess. The lead salts were decomposed with H_2S, the PbS centrifuged off, and the solution aerated free of H_2S. The solution was neutralized to pH 8.2, and excess $Ba(Ac)_2$ was added, plus 4 volumes of cold 95 per cent EtOH. The precipitate was plated directly on thin aluminum plates and the specific activity of the PDP determined by counting and eluting each plate and analyzing for total phosphorus. These results are presented in Table 7. It is to be noted that the above procedure is not a method which accounts quantitatively for the total PDP formed, and thus the values given represent minimal values.

[5] Prepared by the method of Lindberg (6).

A preliminary investigation of these reactions was undertaken with several other tissues. In rat liver, brain, and kidney, the radioactivity disappearing from the pyruvate-lactate pool was accounted for mainly in the Ba-soluble–alcohol-insoluble fraction of the reaction mixtures.

DISCUSSION

The metabolism of pyruvate has been shown to follow a great variety of pathways. Potter and Heidelberger (9) noted that at least eight different means of utilizing pyruvate are present in animal tissues. Among these are conversion to lactate, alanine, serine; condensation to C_4 compounds; and decarboxylation to C_2 and C_1 fragments. In the tumor homogenate system used here, under anaerobic conditions, conversion to lactate was the main pathway. Except for this, all other known reactions concerning the metabolism of pyruvate were shown to be negligible, relative to a new reaction which resulted from a reduction of pyruvate and had a magnitude comparable to the conversion to lactate. Evidence obtained from carrier experiments strongly suggests that propanediol phosphate ($CH_3CHOHCH_2OPO_3H_2$) represents a large part of the reduced products. Further evidence of this identity is being accumulated. In addition, tracer experiments with the radiocarbon-labeled pyruvate indicate that a second component occurs in the Ba-soluble–alcohol-insoluble

TABLE 7

CARRIER EXPERIMENTS WITH PROPANEDIOL PHOSPHATE

The propanediol phosphate was added to the hydrolyzed Ba-insoluble–alcohol-insoluble fraction of tumor homogenate experiments with pyruvate-2-C^{14} incubated 60 min.

Experiment	Sp. act. found counts/min/μm	Theoretical sp. act.* counts/min/μM	Per cent of disapp. pyruv. as PDP/flask
I	100	124	13.4
II	826	635	26.0

* Assuming all the counts in this fraction to be in the form of propanediol phosphate.

fraction, which is hydrolyzed by treatment with acid. Preliminary indications are that this is acetol phosphate ($CH_3COCH_2OPO_3H_2$), which would be a logical intermediate in the reduction of pyruvate to propanediol phosphate. It appears that the reduction of the carboxyl group is preceded by a phosphorylation.

Experiments are now in progress to isolate the proposed intermediate, acetol phosphate, and to evaluate the significance of these reactions to the metabolism of both normal and neoplastic tissues.

SUMMARY

The anaerobic glycolysis system with homogenates of Flexner-Jobling carcinoma was shown to metabolize pyruvate to reduced products other than lactate to an extent almost comparable in magnitude to the conversion to lactate. The reactions were studied with pyruvate-1-C^{14} and pyruvate-2-C^{14}. The metabolism of pyruvate to alanine, serine, to C_4, C_2, and C_1 was shown to be negligible in this system. A new reaction was found in which the pyruvate was converted to propanediol phosphate, with acetol phosphate the probable intermediate. The significance of these findings to the metabolism of normal and neoplastic tissues is being investigated.

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